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Characterizing the Role of Fungal Shape in a Zebrafish Model of Invasive Candidiasis

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CHARACTERIZING THE ROLE OF FUNGAL SHAPE IN A ZEBRAFISH MODEL OF INVASIVE CANDIDIASIS

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Candida albicans is a common hospital-acquired fungal infection, and disseminated disease claims up to one-half of those afflicted. *C. albicans* has a unique ability to differentiate its shape during infection, and this differentiation is thought to be a major virulence factor during invasive infections. Each shape is proposed to have a specialized role: filaments drive tissue invasion and yeast mediate dissemination to the bloodstream. However, it has been difficult to test these hypotheses for two reasons. First, rigorous testing of shape-specific roles requires diverse strategies of shape modulation that restrict the possibility of manipulation-specific artifacts. Second, although connecting shape to function requires simultaneous visualization of shape and location of fungi during infection, this is not feasible in opaque mammalian infection models. Thus, we aimed to more comprehensively analyze how *C. albicans* utilizes shape to disseminate and invade during infection by using a larval zebrafish infection model. This model permitted the use of diverse, complementary strategies to manipulate shape, subsequently allowing us to monitor dissemination, invasion, and overall pathogenesis using intravital imaging of individual fungal cells throughout the host. To control cell shape, we employed three different strategies: filament-specific gene deletion, overexpression of yeast or filament master regulators, and alteration of infection temperature. The effects of these orthogonal

manipulations were consistent, support proposed specialized roles of yeast in dissemination and filaments in tissue invasion and pathogenesis, and indicate conserved mechanisms in zebrafish. Based on the conservation of cell type-specific functions in zebrafish infection, we tested whether either morphotype changes the effectiveness of the other cell type. By infecting fish with a known mix of shape-locked strains, we surprisingly found that mixed infections were associated with additive, but not synergistic, filament invasion and yeast dissemination. Taken together, these findings provide rigorous confirmation of previous hypotheses linking shape and pathogenesis in a new infection model, but also reveal the independent roles of yeast and filaments during disseminated candidiasis. Overall, we hope that characterizing cell type-specific roles during infection can tailor novel therapies to limit both dissemination and pathogenesis, and efficiently target *C. albicans* in patients who would otherwise succumb to such deadly infections.

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TABLE OF CONTENTS

ACKNOWLEDGMENTS ii

LIST OF TABLES vi

LIST OF FIGURES vii

Chapter

1. REVIEW OF THE LITERATURE 1

1.1 Host-Pathogen Interactions: The War Within Our Bodies 1

1.1.1 Pathogen Immune Evasion Through Differentiation 2

1.2 Underappreciated, but Dangerous: Fungal Infections 2

1.2.1 Differentiation of Shape can be Important During Fungal Pathogenesis 5

1.3 The Opportunistic Fungal Pathogen *Candida albicans*: Overview 6

1.3.1 Types of Infection: Mucosal Candidiasis 7

1.3.2 Types of Infection: Systemic Candidiasis 8

1.3.3 Dimorphism as a Major Virulence Contributor 9

1.3.3.1 Environmental Regulation of Morphogenesis 10

1.3.3.2 Genetic Regulation of Morphogenesis 11

1.3.3.3 Downstream Effects of Deletion or Overexpression of Morphology Genes 14

1.3.3.4 Morphogenesis During *In Vitro*/*In Vivo* Infections 16

1.3.4 Other *C. albicans* Virulence Factors 18

1.3.5 *C. albicans* Morphotype Recognition and Response by the Innate Immune System 20

1.4 Important and Relevant Infection Models....21
1.4.1 *In Vitro* Model Systems....21
1.4.2 *In Vivo* Model Systems....23
1.4.2.1 *Mus musculus*: The Mouse....23
1.4.2.2 *Danio rerio*: The Zebrafish....25
1.4.2.2.1 Modeling Mucosal Candidiasis in Larval Zebrafish....26
1.4.2.2.2 Modeling Systemic Candidiasis in Zebrafish....26
1.5 Summary....28
2. CHARACTERIZING THE ROLE OF FUNGAL SHAPE IN A ZEBRAFISH MODEL OF INVASIVE CANDIDIASIS....30
2.1 Introduction....30
2.2 Materials and Methods....33
2.2.1 Zebrafish Care and Maintenance....33
2.2.2 Ethics Statement....33
2.2.3 Fungal Strains and Growth Conditions....34
2.2.4 Engineering of dTomato, iRFP, and NEON Expressing *C. albicans* Strains....35
2.2.5 Microinjection....36
2.2.6 Fluorescence Microscopy....37
2.2.7 Fungal Burden Assay for Colony-Forming-Units (CFUs)....37
2.2.8 Imaging and Quantification of Filament Invasion and Yeast Colonization in Intact Fish....38
2.2.9 Percent Survival Quantification....38
2.2.10 Percent Dissemination Quantification....39
2.2.11 Filament Invasion Analyses....39

2.2.12 Yeast Yolk Level Analyses 40
2.2.13 Neutrophil Quantification of Whole Fish Using Flow Cytometry 40
2.2.14 Neutrophil Quantification of Tail Fin Transection Using Sudan Black Staining 41
2.2.15 Statistical Analysis 41
2.3 Results 42
2.3.1 Yeast Growth Leads to Dissemination While Filamentous Growth Leads to Invasion and Death 42
2.3.2 Temperature Modulation of Morphology Confirms Morphotype-Function Relationship Without Affecting Zebrafish Immune Function 48
2.3.3 Drug-Regulated Overexpression Strains Change Morphology With Doxycycline in Zebrafish 54
2.3.4 Increased Filament Production Moderately Correlates with Increased Mortality, While Increased Yeast Production Does Not Always Correlate with High Dissemination 60
2.3.5 Morphotypes Do Not Synergize to Enhance Infection Outcome During Mixed Infections 62
3. DISCUSSION 70
4. FUTURE DIRECTIONS 77
REFERENCES 85
APPENDIX: *C. albicans* NEON INFORMATION 109
BIOGRAPHY OF AUTHOR 110

LIST OF TABLES

Table 1.1. Environmental Factors Involved in *C. albicans* Differentiation 11

Table 2.1. Strains Used in this Study 35

Table 2.2. PCR Primers Used in this Study 36

LIST OF FIGURES

Figure 1.1. Genetic and environmental regulation of *C. albicans* morphogenesis 12

Figure 2.1. Wildtype infections result in filament growth and invasion of tissue while yeast-locked infections result in yeast growth and dissemination 43

Figure 2.2. Hypofilamentous mutants grow pseudohyphae during infection 44

Figure 2.3. *C. albicans* filaments invade and cause death, while yeast disseminate in larval zebrafish 46

Figure 2.4. Inoculum size is not a determining factor in mortality, but invasion determines death in filament-dominant infections 47

Figure 2.5. Inoculum size and yeast growth are not determining factors in disseminated yeast infections 48

Figure 2.6. Wildtype infection can be manipulated by temperature to produce yeast, dissemination, and prolonged survival at 21°C 49

Figure 2.7. Morphology-specific infection phenotypes are conserved at high and low temperatures 51

Figure 2.8. Neutrophil ratios do not differ significantly between mock and yeast-infected fish at any temperature 53

Figure 2.9. Neutrophil numbers quantified at site of transection are similar at all incubation temperatures 54

Figure 2.10. Regulating overexpression strains TT21 and $NRG1^{OEX}$ with doxycycline is feasible in zebrafish 56

Figure 2.11. Manipulating overexpression strains with doxycycline results in similar morphology, dissemination, and mortality phenotypes as TT21 infections at 33°C 57
Figure 2.12. Manipulating overexpression strains with doxycycline results in similar morphology, dissemination, and mortality phenotypes as TT21 infections at 28°C 58
Figure 2.13. Manipulating overexpression strains with doxycycline results in similar morphology, dissemination, and mortality phenotypes as TT21 infections at 21°C 59
Figure 2.14. Increased filament production moderately correlates with increased mortality, while increased yeast production does not always correlate with high dissemination 61
Figure 2.15. Morphotypes do not affect each other's abilities to disseminate or invade in mixed overexpression strain infections 63
Figure 2.16. Morphotypes do not synergize to enhance dissemination or invasion in mixed hypofilamentous mutant and wildtype infections 64
Figure 2.17. Single and mixed morphology infections of overexpression strains produce similar levels of dissemination and mortality at 33°C and 21°C 65
Figure 2.18. *C. albicans* grows at distinct speeds during altered temperature incubation 66
Figure 2.19. Filaments and invasion do not significantly alter the ability of yeast to disseminate or grow in the yolk 68

Figure 2.20. Yeast do not affect the ability of filaments to kill fish or to invade during mixed infections 69

Figure A.1 NEON insert sequence for the pENO1-NEON-NAT^{R} plasmid 109

Figure A.2 CUG-optimized NEON *C. albicans* 109

CHAPTER 1

REVIEW OF THE LITERATURE

1.1 Host-Pathogen Interactions: The War Within Our Bodies

Humans are home to an incredible population of roughly 10^{14} microbes, outnumbering the total number of human cells by one order of magnitude (10^{13}) (Tannock GW, 1995). These commensal microbes create the flora found on our mucosal surfaces, and aid our bodies in performing functions like food digestion in the gastrointestinal tract (Naidu AS *et al*., 2010). However, our bodies can sometimes become weak from various stressors, and these weaknesses can allow commensal and other non-flora microorganisms to become opportunistic and pathogenic. Humans can be infected by pathogens as a result of bodily function and commensal niche disruptions: for instance, through predisposition from chemotherapeutic drugs, steroids, or antibiotics, or the interruption of skin and other surface barriers (e.g., cuts, burns, bites) (Parham P, 2009). As pathogens invade these disrupted and dysregulated areas, the immune system must react in order to keep us alive. Tissues in the infiltrated area signal for immune cells to approach the infection and trigger an inflammatory response. These host-pathogen interactions result in a critical war, where survival of the host depends upon destruction of the pathogen.

The human immune system consists of two arms: the first-responder innate system, and the highly specialized adaptive system. Each arm is important in both response to initial infection and protection from recurrent infections. The innate immune arm is termed non-specific and encompasses phagocytic cells like neutrophils and macrophages that engulf pathogens and activate signaling to other immune cells to establish an inflammatory response cascade (Medzhitov R and Janeway CA, 1997). In contrast, the adaptive arm is more highly specialized, consisting of T and B cells that differentiate into effector and memory cells to produce antibodies and elicit stronger, quicker responses in recurrent infections (Palm NW and Medzhitov R, 2008). Both antigen processing and antibody production are notable areas of interest in vaccination

development for many infectious diseases (Kaech SM *et al*., 2002). Overall, both the innate and adaptive systems are highly integrated and work together to process antigens and quickly resolve infections. Breakdowns in either system can result in recurring, chronic infections, or in severe cases, death of the host. It is therefore important that host defenses persist and overwhelm pathogen viability.

1.1.1 Pathogen Immune Evasion Through Differentiation

Strikingly, many viral, bacterial, and fungal pathogens have acquired the ability to differentiate their shape, cell walls, or gene/protein expression during infection to evade immune recognition and survive in the host. For instance, herpes simplex virus (HSV) can induce production of viral protein VP16 to activate the switch from latent to lytic phase in trigeminal ganglion neurons, allowing repeated transmission of the virus to other neural cells (Thompson RL *et al*., 2009). Some bacteria, such as *E. coli* and *P. aeruginosa*, initiate growth of thick polysaccharide pods, similar to biofilm components, inside host cells to avoid both antibiotic treatment and immune recognition (Anderson GG *et al*., 2003, Garcia-Medina R *et al*., 2005). Further, many human fungal pathogen species can physically change shape to avoid immune recognition or to invade tissue (Chai LYA *et al*., 2009). Fungi, such as *Histoplasma capsulatum*, are also able to vary their cell wall composition so that normally recognized pathogen-associated molecular patterns (PAMPs), such as β-glucan, can be masked and thereby avoid immune recognition (Rappleye CA *et al*., 2007, Chai LYA *et al*., 2009). Taken together, these diverse mechanisms are used by pathogens to evade immune recognition and cause infection, and may be used as potential targets for novel therapeutic development.

1.2 Underappreciated, but Dangerous: Fungal Infections

When most people hear “infection”, they usually think of the major viral and bacterial disease outbreaks recently portrayed in the media: “Deadliest ever outbreak of Ebola virus” (Cullinane S and Thompson N, 2014), “Antibiotic Resistant Gonorrhea Spreading Rapidly” (Sifferlin A, 2017), “*E. coli* outbreak in 11 states linked to store-bought chopped romaine lettuce”

(Phillips K, 2018). These outbreaks have rightfully garnered interest from people, but this interest has left other infectious agents underappreciated, including many relevant fungal pathogens. In general, fungal pathogens can detrimentally affect plants, animals, and even humans, causing both ecological and economical strain across the globe (Fisher MC *et al*., 2012).

In the context of plant disease, fungi of the phylum Ascomycota have wiped out entire forests, and therefore ecosystems, by causing blight in Dutch Elm and Chestnut trees in the United States alone (Schlarbaum SE *et al*., 1997). Further, the causative agent of potato blight, *Phytophthora infestans*, infamously decimated potato crops in 1845 Ireland, effectively killing over one million Irish (Yoshida K *et al*., 2013). Thus, fungal infections of plants have rapidly decimated ecosystems and food sources for both animals and humans.

Fungal infections have rapidly infected and decimated many animal species. In 2007, officials in New York State discovered an outbreak of *Geomyces destructans*, the causative agent of white nose syndrome, in hibernating bats tightly clustered in caves. This fungal infection leads to high mortality and destroyed over 70% of bat species in affected areas, causing near extinction in the little brown bat species alone (Frick WF *et al*., 2010). Another fungus discovered in 2006, *Ophidiomyces ophidiodiicola*, causes the infectious snake fungal disease that can affect all snake species, and recently killed 50% of a timber rattlesnake population in New Hampshire alone (Burbrink FT *et al*., 2017, Clark RW *et al*., 2011). Other fungi have caused major declines in crayfish (*Aphanomyces astaci*), bees (*Nosema* species), soft corals (*Aspergillus sydowii*), and tilapia (*Aphanomyces invadans*), suggesting that pathogenic fungi are a major threat to many animal species throughout the world (Fisher MC *et al*., 2012).

In the context of human disease, fungi cause superficial infections in both healthy and immunocompromised people, as well as more invasive disease in immunocompromised patients. Superficial infections of the skin and other tissues are usually not life-threatening, while

invasive, deep-seated infections can not only maim patients, but are often lethal (Brown GD *et al*., 2012).

Some common fungal infections cause damage to superficial tissues in both immunocompetent and immunocompromised patients. For instance, dermatophytes such as *Tinea capitis* and *Tinea corporis* cause ringworm, which is easily treatable and resolves quickly after initiation of antifungal treatment (Kelly BP, 2012). Further, the causative agent of common Athlete's foot, *Tinea pedis*, is also easily treated with over-the-counter antifungals (Ogasawara Y *et al*., 2003). Thus, these common skin-related fungal infections are usually relatively easy to treat and eliminate.

Major invasive fungal pathogens have emerged in recent years that cause high morbidity and mortality in patients, especially in those with compromised immune systems due to chemotherapy/corticosteroid use, transplant therapy, or coinfection with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) (Walsh TJ and Groll AH, 1999). Invasive infections caused by environmental fungi like *Aspergillus fumigatus*, *Pneumocystis jirovecii* (PCP), *Cryptococcus neoformans/gatti*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Coccidioides* sp. result in mortality rates as high as 90% in HIV/AIDS patients alone (Dagenais TRT and Keller NP, 2009, Morris A *et al*., 2004, Charlier C *et al*., 2005, Chapman SW *et al*., 2008, Huang JY *et al*., 2012, Loulergue P *et al*., 2007). These fungal infections, in particular aspergillosis, cryptococcosis, and histoplasmosis, can cost between $6,000 to $158,000 per hospitalization, placing an enormous economic burden on the patient (Wilson LS *et al*., 2002).

Similar to these pathogenic environmental fungi, the human fungal pathogen *Candida* is the fourth most common nosocomial infection in the country behind bacteria, and the most common fungal pathogen found in this setting, resulting in high mortality rates in patients with invasive infections (Wisplinghoff H *et al*., 2004). *Candida albicans* is the predominant species that causes invasive and mucosal infections in humans (Perlroth J *et al*., 2007). Other species of

Candida that cause invasive disease in humans include sp. *glabrata*, *parapsilosis*, *krusei*, and *dubliniensis* (Krcmery V and Barnes AJ, 2002). Interestingly, *Candida auris* has recently emerged as a global threat to human health, with an already high rate of resistance to multiple antifungal treatments (Chowdhary A *et al*., 2014, Lockhart SR *et al*., 2016). In general, hospitalization for invasive candidiasis infections can amount to nearly $45,000 per patient, and an estimated $216-$281 million per year total for patients in the United States alone (Rentz AM *et al*., 1998). Thus, even with current antifungal treatments, these invasive fungal infections require hospitalization and have high rates of mortality, requiring scientists to rapidly search for new therapeutics to relieve economic burden and overcome treatment resistance.

Overall, fungal infections in humans, animals, and plants can have negative implications that place added burden and stress on the economy and the ecosystem. Therefore, it is crucial that we begin to regard fungal pathogens in a more serious context, as these infections are as important and detrimental as other microbial pathogens.

1.2.1 Differentiation of Shape can be Important During Fungal Pathogenesis

Some human fungal pathogens alter their shape by using dimorphic transitions, and these different forms can help contribute to infection. For instance, *Cryptococcus neoformans* infects humans as a unicellular yeast/spore form; however, it can grow non-pathogenically as a multicellular filament in the environment, and will bud yeast cells that are eventually inhaled by people (Wickes BL *et al*., 1996). *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Paracoccidiodies brasiliensis* all form non-pathogenic filamentous molds in the environment, but once inhaled, the temperature shift between environment and body will induce filament transition to yeast, which then infect the host and cause pathogenesis (Retallack DM and Woods JP, 1999, Medoff G *et al*., 1987). In contrast, other pathogenic fungi grow filaments that are important for pathogenesis once inside the body. For instance, *Aspergillus fumigatus* is inhaled as unicellular conidia (similar to yeast), but once inside the lungs, the fungus will germinate into long filaments that invade host tissue and disseminate into the bloodstream

(McCormick A *et al*., 2010). *Candida albicans* and *Candida dubliniensis* also form filaments from yeast cells that are thought to be important for deep-seated tissue invasion and damage of host organs and other tissues (Gow NAR *et al*., 2002, Sullivan D and Coleman D, 1998). Overall, these shape-shifting fungi suggest that dimorphism is important for pathogenesis during invasion of the host and disease spread.

Some fungal species do not germinate, but rather cause infection as a singular morphotype. Non-*albicans Candida* species (NACS), such as sp. *glabrata* and *auris*, cause infection in the yeast form, without the need of a transition to the filamentous state (Silva S *et al*., 2011, Satoh K *et al*., 2009). *C. parapsilosis* also infects as a yeast, but can grow as pseudohyphae under certain conditions *in vitro* (Trofa D *et al*., 2008). *Pneumocystis jiroveci*, the causative agent of *Pneumocystis* pneumonia (PCP), an AIDS-defining illness, grows and infects as a cyst (Frenkel JK, 1976, Thomas CF and Limper AH, 2007). In addition, rare but infectious fungi like *Geotrichum* sp. and *Hansenula anomala* also cause invasive infections in severely immunocompromised patients without germinating (Miceli MH *et al*., 2011). Thus, the necessity of dimorphism in the context of pathogenesis is important in many fungal species, but not all. Overall, the presence of single morphotypes and the differentiation of shape in human fungal pathogens remain unique and important features of these infections, and could be considered as potential therapeutic targets.

1.3 The Opportunistic Fungal Pathogen *Candida albicans*: Overview

Candida albicans is a major opportunistic fungal pathogen that can cause both invasive and superficial infections in humans. This pathogen is generally found as a commensal organism in the oral/gastrointestinal tract, on the skin, and in the genitourinary tract of the human body (Soll DR, 2002). However, when immunodeficiency occurs through drug therapy or HIV infection, the fungus can become pathogenic and wreak havoc in the host. Worldwide, *C. albicans* causes roughly 12 million cases per year of mucosal thrush and esophageal infections. Less prevalent, but deadlier, bloodstream infections add up to about 400,000 cases per year (Pal M, 2017). This

pathogen is the predominant *Candida* species identified in both invasive and mucosal infections (Perlroth J *et al*., 2007). In hospital-associated infections, the fungus is able to enter the body through medical device implantation (e.g., catheters, pacemakers) as well as sterilization mishaps involving surgical equipment (Kojic EM and Darouiche RO, 2004). Further, *C. albicans* on the skin can enter the body via burn wounding (Gupta N *et al*., 2005). Overall, this fungus wreaks havoc in millions of immunocompromised patients by causing superficial and invasive infections through pathogenic activation, and non-commensal entrance into the body through equipment mishaps and barrier disruptions.

Resistance to antifungal treatment has been and continues to be a major issue encountered with many clinical isolates. Many first-line agents have reported at least some amount of resistance with *C. albicans*, including the most commonly used antifungals fluconazole, caspofungin, and amphotericin B (Dixon DM and Walsh TJ, 1996, Balashov SV *et al*., 2006). Thus, the vast capabilities of this fungus to cause infection and persist during treatment remains a major issue in medicine.

1.3.1 Types of Infection: Mucosal Candidiasis

Mucosal candidiasis is characterized by the colonization and invasion of *C. albicans* into the mucosal, epithelial tissues of the body, resulting in many symptoms depending on the area of infection. Oral and esophageal candidiasis usually result in redness, soreness, and white patches in the mouth/throat (Lynch DP, 1994). These infections are commonly found in babies, the elderly, and those with HIV and other chronic syndromes (Akpan A and Morgan R, 2002). Vulvovaginal candidiasis (VVC), more commonly known as a vaginal yeast infection, infects an estimated 75% of women worldwide at least once in their lifetime (CDC, 2015). Vulvovaginal candidiasis is characterized by vaginal itching, soreness, pain during intercourse, and abnormal discharge, and can cause recurrent infection in 5-10% of women. Risk factors for this infection include antibiotic use, pregnancy (or estrogen hormonal changes), and sexual activity, and affect both immunocompetent and immunocompromised individuals (Goncalves B *et al*., 2015).

Thus, depending on the area of colonization and patient background, mucosal infections can affect multiple areas of the body in a diverse group of patients.

Mucosal candidiasis infections are rarely life-threatening, but can be used as important markers for more severe, underlying conditions. For instance, oral candidiasis is one of the first indicators of HIV infection and is usually found in up to 95% of HIV patients before AIDS overpowers the body (Conti HR and Gaffen SL, 2010). Further, people with inherited immunodeficiency diseases, such as severe combined immunodeficient disease (SCID) or chronic granulomatous disease (CGD), can continually display symptoms of mucosal infection, termed chronic mucocutaneous candidiasis (CMC), resulting in rare symptom relief without antifungal treatment (Puel A *et al*., 2013, Kirkpatrick CH, 2001). Thus, mucosal *C. albicans* infections can affect any individual, and remain important in the diagnosis of severe, underlying conditions.

1.3.2 Types of Infection: Systemic Candidiasis

Systemic, bloodstream infections of *C. albicans* usually occur in hospital settings and are accompanied by high mortality rates, up to 50% in some cases, even with antifungal treatment (Gudlaugsson O *et al*., 2003). These infections normally occur when mucosal barriers are disrupted in the body, such as those that occur during surgical procedures or implantation of plastic medical devices, allowing fungal cells to escape into the bloodstream (Ramage G *et al*., 2006). Systemic infections spread fungi to many organs, and lesions can sometimes be found in the kidneys, brains, and even hearts of patients (Parker JC *et al*., 1976). Clinical symptoms are similar to septic shock, where fever and inflammation increase; however, most people who acquire these infections are usually simultaneously afflicted with another medical condition, making early diagnosis difficult (CDC, 2015).

Diagnosis of candidemia is challenging, although multiple methods can be used. Blood cultures may take up to 5 days to grow with only a 50% sensitivity rate, which is not ideal in the context of treatment initiation. However, a novel nanodiagnostic panel, T2Candida, uses T2

magnetic resonance to detect different species of *Candida*, including *C. albicans*, directly from blood samples in a shorter timescale with a sensitivity of 89% (Clancy CJ *et al*., 2018). Thus, with both detection models, faster diagnosis and treatment initiation can be achieved to potentially improve patient care.

Candidemia treatment must begin rapidly after diagnosis or as prophylaxis while waiting on diagnosis, and usually consists of fluconazole as first-line therapy due to its high safety profile (Charlier C *et al*., 2006). Echinocandins such as caspofungin are also regularly used due to their broad-spectrum activity against many *Candida* species other than just sp. *albicans* (Wagner C *et al*., 2006). In very severe, invasive bloodstream infections, amphotericin B is administered, but is associated with extreme side effects, including toxicity in the kidneys (Gallis HA *et al*., 1990). Thus, multiple antifungals are available for treatment of invasive candidiasis, although the toxicity, and the high prevalence of resistance, in some reveals a need for newer, less damaging, therapeutics.

Overall, although systemic *C. albicans* infections are less common than mucosal infections, they remain lethal to nearly half of patients who acquire them, even with antifungal treatment, revealing their importance as a major nosocomial pathogen.

1.3.3 Dimorphism as a Major Virulence Contributor

Candida albicans is able to physically switch between two major shapes, yeast and hyphae (filaments), during infection, and this switch is thought to be a major virulence factor that enhances pathogenesis (Culter JE, 1991). In general, both yeast and filaments grow via mother-daughter budding; however, filaments continue to grow long germ tubes that connect multiple cells together through septa. In some cases, pseudohyphae may also grow, and while these cells are labeled “filamentous”, the structures of their germ tubes differ slightly compared to filaments (Sudbery PE *et al*., 2004). In general, the switch between yeast-filament or filament-yeast growth is heavily regulated by multiple environmental and genetic factors, both *in vitro* and *in vivo*. As a major pathogenic fungus in humans, *C. albicans* can adapt to the body and use

multiple environmental cues to spatially and temporally alter its shape. This well-timed switch can drive the fungus from a commensal state into a pathogenic state.

1.3.3.1 Environmental Regulation of Morphogenesis

Candida albicans is able to differentiate from a yeast to filamentous shape due to multiple environmental factors *in vivo*. As yeast cells grow in the host, multiple filament-inducing factors can influence their germination. In human tissue, high levels of carbon dioxide and low levels of oxygen (hypoxia) present a suitable environment for germination to initiate via activation of multiple filament-inducing biochemical pathways (Klengel T *et al*., 2005). Further, increases in human hormones, such as estradiol and progesterone, activate filament growth, and changes in these hormone levels in humans may be partially responsible for the induction of vaginal candidiasis (Kinsman OS *et al*., 1988). It is important to note that in the intact host, *C. albicans* cells are simultaneously provoked by multiple environmental cues, and must choose which cues to obey to balance commensalism and pathogenesis. Thus, depending on the environment in the host, transitions between yeast and filament forms can occur under multiple conditions and induce pathogenesis.

C. albicans has adapted to growing as a yeast or a filament form under certain environmental conditions *in vitro*, summarized in Table 1. Many of these conditions have been exploited to filament- or yeast-bias the fungal population to understand certain aspects of fungal infection, such as phagocytosis or tissue invasion. For instance, growing yeast at a high temperature (37°C) will induce filamentous growth, especially in the presence of serum. This environment is necessary for studies involving mammalian cells, and can be used to study how host immune cells respond to filaments or the process of germination (Rubin-Bejerano I *et al*., 2003). In contrast, infections of mice and other animals transpire using the yeast form due to its small size and ease of handling. Thus, growing the fungus overnight at a low temperature (30°C) with nutrients such as glucose and amino acids included in the media is sufficient to induce yeast growth for animal infection (Nantel A *et al*., 2002). Interestingly, starving yeast of

glucose can induce germination as a survival mechanism *in vitro* (Ernst JF, 2000). For instance, when starved yeast cells are incubated in media containing N-acetylglucosamine, a carbon source abundant in the gastrointestinal tract, filament growth is induced due to a lack of other nitrogen and carbon sources (Du H *et al*., 2015, Kumar MJ *et al*., 2000, Brown AJP and Gow NAR, 1999). Overall, multiple environmental factors can affect the ability of *C. albicans* to regulate its shape *in vitro*.

Environmental Factors Involved in *C. albicans* Differentiation	
Yeast	**Filaments/Pseudohyphae**
Low pH (4.0) High cell density Low temperatures (<30°C) Glucose	High pH (6.0) High temperatures (37°C) Serum High CO_2 Hypoxia Nutrient starvation N-acetylglucosamine

Table 1.1 Environmental Factors Involved in *C. albicans* Differentiation. Adapted from Sudbery PE et al., 2004.

In general, yeast and filament growth is regulated by many functions *in vivo* and *in vitro*, and can be altered by changes in the environment. This ability to change form has helped it to endure various environmental cues in the body to balance growth as a commensal or activation for pathogenesis.

1.3.3.2 Genetic Regulation of Morphogenesis

Major biochemical pathways, such as the cAMP-PKA and MAPK pathways, regulate expression of both yeast- and filament-specific genes, as summarized in Figure 1.1. The GTP-binding protein Ras can stimulate both MAPK and cAMP-PKA pathways when induced by environmental stressors, such as starvation, temperature changes, and presence of serum (Biswas S *et al*., 2007). Downstream activation of the MAPK pathway stimulates the expression of the filament-inducing *CPH1* gene, whereas activation of the cAMP-PKA pathway stimulates downstream expression of filament-specific *EFG1* and *UME6* genes (Leberer E *et al*., 2008). These genes work in feedback loops with yeast-specific genes, such as *NRG1* and *TUP1*, to

balance dimorphic transition (Murad AMA *et al*., 2001a/b). Thus, multiple pathways and genes regulate the ability of *C. albicans* to change its shape.

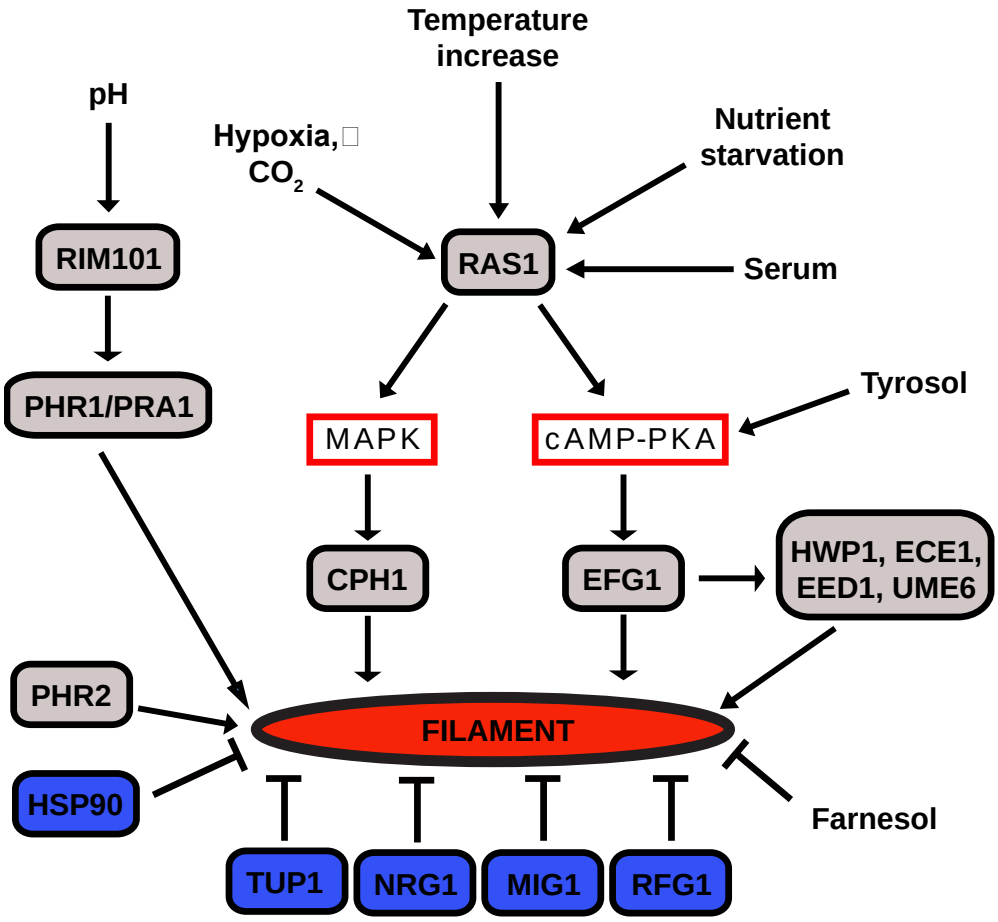

Figure 1.1 Genetic and environmental regulation of *C. albicans* morphogenesis. Multiple environmental factors activate filamentation through MAPK and cAMP-PKA biochemical pathways (outlined in red) and filament-specific gene expression (grey boxes). Transcription factors like *TUP1*, *NRG1*, *MIG1*, and *RFG1* (blue boxes) also negatively regulate filament-specific genes and promote yeast growth. Arrows represent activation, blunt arrows represent inhibition.

In a study analyzing the importance of *EFG1* and *CPH1* during filament induction, the authors found that *EFG1* seemed to be the major influencer of filamentous growth, with *CPH1* having a lesser role in activation (Braun BR and Johnson AD, 2000). This idea stems from multiple analyses showing that multiple downstream filament genes, such as *ECE1* and *HWP1*,

were not significantly affected by *CPH1* deletion when *EFG1* was still present, whereas *EFG1* deletion resulted in diminished expression of many filamentous genes (Braun BR and Johnson AD, 2000). However, deletion of *CPH1* results in defective filament growth, indicating that its presence is important in some form for filament growth (Braun BR and Johnson AD, 2000). Thus, the cAMP-PKA pathway and *EFG1* seem to be the more important filament-inducing regulators, although *CPH1* does contribute to filament growth.

pH and temperature are two major regulators of filament morphogenesis, presumably due to the potential pH and temperature changes the fungus may encounter in different niches throughout the host (Burton AC, 1935, Schmaljohann D, 2006, De Bernadis F *et al*., 1998). The zinc finger transcription factor *RIM101* regulates filamentous growth due to changes in environmental pH. This factor regulates expression of *PHR1* and *PRA1*, both important in the induction of filamentous growth under alkaline conditions (Davis D *et al*., 2000). A second PHR gene, *PHR2*, is also important for filamentous growth in the absence of *PHR1* (Muhlschlegel FA and Fonzi WA, 1997). In the context of temperature, heat shock proteins are important in regulation of morphogenesis. An important heat shock protein, Hsp90p, has been implicated in regulation of the Ras-PKA pathway, and generally represses the filament-inducing pathway under normal conditions. However, during an increase in temperature, Hsp90p relieves its repression of the Ras-PKA pathway and filamentation initiates (Shapiro RS *et al*., 2009). Thus, both pH and temperature responsive genes are necessary to activate filamentous growth of *C. albicans* under certain conditions.

Similar to some bacteria, *C. albicans* is able to secrete quorum-sensing molecules that affect gene expression and dimorphic transitions in both high- and low-density environments or for activation of tissue invasion. For instance, the quorum-sensing molecule tyrosol promotes filamentous growth through activation of the cAMP-PKA pathway, and regulates filament-specific *EED1* expression (Chen H *et al*., 2004, Alem MAS *et al*., 2006). In contrast, a second quorum-sensing molecule, farnesol, is produced by yeast in high density conditions and inhibits

filamentous growth via inhibition of the Ras-PKA pathway and therefore inhibition of *EFG1* (Davis-Hanna A *et al*., 2007). Thus, *C. albicans* uses quorum-sensing molecules to regulate gene expression and shape based on fluctuations in cell-population density.

Filament growth is also negatively regulated by multiple transcription factors, including *TUP1*, *NRG1*, *RFG1*, and *MIG1*, which instead promote yeast growth. *TUP1* and *NRG1* co-regulate the filament-specific genes *HWP1*, *ECE1*, *ALS8*, *UME6*, and *HYR1* by repressing their expression and therefore yeast-filament transition (Murad AMA *et al*., 2001a/b, Kadosh D and Johnson AD, 2001). *RFG1* also represses filament-specific *ECE1* and *HWP1* gene expression (Kadosh D and Johnson AD, 2001). Thus, multiple factors are able to regulate filament inhibition and promotion of yeast growth under certain conditions. Overall, *C. albicans* morphogenesis is controlled by many positive and negative regulators that influence and balance the necessity of dimorphic switching during infection.

1.3.3.3 Downstream Effects of Deletion or Overexpression of Morphology Genes

Genetic manipulation of morphological genes can affect downstream gene expression for virulence, cell wall maintenance, and metabolism in *C. albicans*. *EFG1* is important in the regulation of multiple virulence factors, including many adhesins and hydrolytic enzymes. For instance, the expression of adhesin gene *ALS1* is diminished in a strain lacking the filament-specific *EFG1* gene (*efg1*Δ/Δ), suggesting that *EFG1* regulates *ALS1* expression (Fu Y *et al*., 2002). Further, some hydrolytic enzymes are also regulated by *EFG1*, and display diminished expression when these genes are deleted (Kumamoto CA and Vinces MD, 2005). Other filament-specific virulence and cell wall genes, including *HYR1*, *ECE1*, *HWP1*, and *RBT1*, are also heavily regulated by *EFG1* and result in diminished expression in the absence of the gene (Braun BR and Johnson AD, 2000). In addition, *EFG1* was also found to upregulate fatty acid desaturation during hypoxic conditions, as an *efg1*Δ/Δ mutant had a lower presence of unsaturated fatty acids (Setiadi ER *et al*., 2006). This suggests that *EFG1* is not only involved in downstream filament- and virulence-specific gene regulation, but also metabolic regulation, and

that a deficiency in *EFG1* expression affects fatty acid metabolism, which is important for membrane fluidity in both yeast and filaments (Shiradhone AB *et al*., 2018). Thus, *EFG1* is a major regulator of multiple downstream genes necessary for *C. albicans* virulence, filament production, and some metabolic functions, and a defect in expression results in multiple downstream dysregulations and defects.

The filament-specific genes *EED1* and *UME6* are also important in the regulation of many downstream genes important for *C. albicans* growth and virulence. In a strain lacking *EED1* (*eed1*Δ/Δ), *UME6* expression was downregulated, suggesting that *EED1* regulates *UME6* expression. However, during ectopic expression of *UME6*, multiple *EED1*-regulated cell wall and oxidative stress protection-associated genes are induced, including *ECE1*, *HYR1*, *SOD5*, and *HGC1.* This suggests that *UME6* acts downstream of *EED1* to regulate the expression of these genes (Martin R *et al*., 2011, Carlisle PL and Kadosh D, 2012). Unsurprisingly, as germination initiates *in vitro*, *UME6* expression levels increase while the filament-repressing *NRG1* expression decreases. Further, *NRG1* overexpression results in a repression of *UME6* expression, thus suggesting that these transcription factors function in a regulatory loop that coordinates control of filament-specific target genes in response to multiple filament-inducing cues (Banerjee M *et al*., 2008).

Both *NRG1* and *TUP1* are well-established negative regulators of many filament-specific genes, including *EFG1*, *CPH1*, *EED1*, and *UME6*, as previously mentioned (Kadosh D and Johnson AD, 2005). Surprisingly, inactivation of both of these genes results in an increase of expression of 9-13% of *C. albicans* genes, suggesting that these transcription factors are major negative regulators in *C. albicans* (Murad AMA *et al*., 2001a). Further, many of these genes are not only involved in filament processes, but also metabolic processes, such as alcohol dehydrogenase (*ADH1*), galactokinase (*GAL1*), and malate dehydrogenase (*MDH1*) genes, suggesting that *NRG1* and *TUP1* can downregulate processes separate from filament growth

and virulence (Murad AMA *et al*., 2001a). Thus, *NRG1* and *TUP1* are important in the balance of dimorphic transition as well as some metabolic activation during *C. albicans* growth.

Overall, both filament- and yeast-inducing genes regulate downstream factors for virulence, cell wall maintenance, and metabolism, and dysregulations in these morphogenetic genes can result in up- or down-regulation of genes necessary for growth in multiple conditions. Thus, morphogenetic genes are not only necessary for yeast and filament growth, they are important in the regulation of many general activities in the fungus.

1.3.3.4 Morphogenesis During *In Vitro*/*In Vivo* Infections

Yeast and filaments have been long-hypothesized to have important roles during invasive candidiasis. Current data suggests that the yeast form is important for bloodstream spread of the infection while filaments are thought to induce tissue invasion, leading to damage of host tissue and death of the host (Gow NAR *et al*., 2011) These hypotheses have come from a large accumulation of *in vitro* and *in vivo* infection studies using multiple genetically-manipulated fungal strains that filament- or yeast-bias the population, thus permitting the study of individual morphotypes and their roles during infection.

Multiple genes along the MAPK and cAMP-PKA pathways have been knocked out or overexpressed in strains to understand the role of filaments during infection (Shareck J and Belhumeur P, 2011). Mutants of filament-specific genes *EFG1*, *CPH1*, *EFG1/CPH1*, *EED1*, and *UME6* (*efg1*Δ/Δ, *cph1*Δ/Δ, *efg1*Δ/Δ/*cph1*Δ/Δ, *eed1*Δ/Δ, and *ume6*Δ/Δ) have shown marked reduction in invasion, damage of host tissue, and mortality in multiple animal models (Lo HJ *et al*., 1997, Martin R *et al*., 2011, Banerjee M *et al*., 2008, Brothers KM *et al*., 2011). Further, during *in vitro* infections, a mutant of both *EFG1* and *CPH1* (*efg1*Δ/Δ/*cph1*Δ/Δ) was also found to have reduced capabilities to invade and damage endothelial cells (Phan QT *et al*., 2000). These strains predominantly grow as yeast, and lack the ability to grow as true filaments, suggesting that the filamentous form is important for invasion and virulence during infections. Complementary to this idea, overexpression of *UME6* results in filament-specific growth and a

high rate of mortality and kidney tissue damage in a disseminated mouse infection model (Carlisle PL *et al*., 2009). Overall, these data suggest that genes important for filamentous growth are also important during pathogenesis.

Similar to filaments, multiple genes important for yeast growth have been knocked out or overexpressed to study the role of yeast during infection. Overexpression of *NRG1* produces a morphologically-stable yeast-locked strain that is attenuated in virulence, but is still able to disseminate from the bloodstream to tissues and organs in a mouse infection model (Saville SP *et al*., 2003). Similarly, the yeast-locked attenuated virulence strain *efg1*Δ/Δ/*cph1*Δ/Δ produces dissemination *in vivo* (Bendel CM *et al*., 2003). These data suggest that yeast are important for disseminated infection. Alternatively, mutants in the yeast-specific genes *NRG1* and *TUP1* (*nrg1*Δ/Δ and *tup1*Δ/Δ) result in constitutive filamentous growth *in vitro*, but reveal attenuated virulence *in vivo* in a systemic mouse infection model (Murad AMA *et al*., 2001b, Braun BR *et al*., 2000). This data suggests that the yeast form may also confer a virulence component, presumably through infection spread. This idea is complemented by studies in which the pescadillo (*PES1*) gene, important for filament transition to yeast, is deleted (*pes1*Δ/Δ), resulting in constitutive filament growth and attenuated virulence in an intravenous mouse model and a wax moth model (Uppuluri P *et al*., 2012, Shen J *et al*., 2008). Overall, these data suggest that genes specific for yeast growth are also important for pathogenesis and may be necessary for commencement of disease spread.

Studies observing yeast- and filament-specific strains have suggested that both morphotypes are important for virulence, and that deletions of morphotype-specific genes can cause attenuated virulence for both shapes. For instance, a yeast-locked strain that disseminates to tissue in an intravenous mouse infection model confers attenuated virulence compared to a wildtype infection (Saville SP *et al*., 2003). Similarly, experiments using filament-locked mutants also confer attenuated virulence compared to a wildtype infection (Murad AMA *et al*., 2001b, Shen J *et al*., 2008). This attenuation in virulence derived from yeast- and

filament-locked infections suggests that either the simultaneous presence of both morphotypes or a transition between morphotypes is necessary for virulent pathogenesis. However, current models have yet to address how these morphotypes affect one another during infection and therefore these hypotheses remain as such. Complementary to these ideas, both yeast and filaments have been isolated from peripheral blood smears in systemically infected patients (Nadir E and Kaufshtein M, 2005). Although the interactions between the two remain unknown in this setting, it suggests that the presence of both forms is essential for causing systemic infection in humans. Overall, these studies suggest that dimorphism during infection is important for invasion and disease spread of *C. albicans* throughout the body. Thus, the ability to change shape appears to be a major virulence factor that affects how the fungus causes pathogenesis in humans.

1.3.4 Other *C. albicans* Virulence Factors

Although dimorphism is thought to be a major virulence factor during candidiasis, other secretory and adhesive components of the fungus can contribute to its virulent state. These components may be coregulated with genes necessary for morphology, and, as stated earlier, defects or overexpression of morphology genes may affect how these proteins are produced and secreted for adhesion and damage to host tissues. Thus, it is important to understand the links between other virulence factors *C. albicans* produces and dimorphic transitions to adequately discern virulence and pathogenesis during invasive infections.

The *C. albicans* cell wall is composed of multiple carbohydrates, proteins, and sugars that confer protection of the fungus and adherence to host tissues (Netea MG *et al*., 2008). Adhesins produced on the cell surface of yeast and filamentous *C. albicans* promote attachment to epithelial and endothelial tissues in the host. Some of these adhesins are regulated downstream of major filament-specific genes, including *EFG1* and *CPH1*, and deletions of these genes will not only have defects in filamentation, but also defects in adhesion (Fu Y *et al*., 2002, Tsuchimori N *et al*., 2000, Fan Y *et al*., 2003). In a strain lacking just the adhesin *ALS1* gene

(*als1*Δ/Δ), attenuated adherence and fungal burden occur early in a murine oral infection model, but recovered burdens are comparable to wildtype strains as infection persists. Further, although this adhesin is filament-specific, this strain does not have a filament defect, suggesting that other genes necessary for filament growth and adhesion are upregulated in the absence of this protein (Kamai Y *et al*., 2002). Another adhesin regulated by *EFG1*, Als3p, is important for adherence to cadherins in host cells, initiating endocytosis by epithelial or endothelial tissues (Phan QT *et al*., 2007). Absence of this adhesin *in vitro* results in reduced virulence and damage to epithelial cells, suggesting that this adhesin is required for filament invasion (Almeida RS *et al*., 2008). Hyphal wall protein 1 (Hwp1p) also stimulates adhesion to endothelial cells and absence of its expression results in defective germ-tube formation and markedly reduced virulence in mice (Tsuchimori N *et al*., 2000). Taken together, multiple adhesion molecules are important in adherence to host tissues and initiation of invasion, and are closely regulated with important morphology-specific genes, both *in vitro* and *in vivo*.

C. albicans can also secrete hydrolytic enzymes regulated by morphology-specific genes during infection that contribute to damage of host tissues and cells. The secreted aspartyl proteinases (SAPs) specifically target and degrade host surface and extracellular matrix proteins such as collagen, laminin, fibronectin, and mucin (Schaller M *et al*., 2005). In general, deletions of SAPs 1-6 result in attenuated virulence in both mouse and guinea pig infection models (Hube B *et al*., 1997, Sanglard D *et al*., 1997). SAPS1-3 and 6 have also been expressed in lesions from human oral candidiasis, while SAP2 is necessary for invasion during vaginal candidiasis in a rat model (Schaller M *et al*., 1999, De Bernadis F *et al*., 1999). Another hydrolytic enzyme, phospholipase, is also secreted from the fungus during infection. Phospholipase B1 (PLB1) is secreted at the hyphal tip during invasion and a mutant strain with a knockout of the *PLB1* (*plb1*Δ/Δ) gene is significantly attenuated in virulence and disease spread in both a bloodstream and an oral-intragastric murine infection model (Ghannoum MA, 2000). Hence, multiple hydrolytic enzymes, regulated by filament-specific genes, are necessary

for damage of host tissues and invasion both *in vivo* and *in vitro*.

Recently, a new toxin produced by *C. albicans* was discovered that induces damage to epithelial cell membranes *in vivo*. The cytolytic peptide candidalysin is produced by *C. albicans* filaments through the filament-inducing extent of cell elongation 1 (*ECE1*) gene and acts to permeabilize host epithelial cells via calcium influx. Absence of this toxin during infection results in attenuated damage *in vitro* and in an oral murine model, as well as attenuated virulence in a zebrafish model (Moyes DL *et al*., 2016). This filament-specific toxin, similar to the hydrolytic enzymes, is important for host cell damage and invasion. Taken together, *C. albicans* possesses a multitude of virulence factors co-regulated by important morphogenic genes that allow the fungus to damage tissue and survive inside the host.

1.3.5 *C. albicans* Morphotype Recognition and Response by the Innate Immune System

Both yeast and filaments are recognized by multiple innate immune cell types, including macrophages, neutrophils, and dendritic cells. These immune cells can specifically recognize fungal cell wall components through multiple receptor types, resulting in phagocytosis of the pathogen (Netea MG *et al*., 2008). Neutrophils are normally the first responders to these fungal infections, with capabilities in killing both yeast and filaments. Yeast cells are easily phagocytosed and degraded in the phagolysosome by multiple antimicrobial peptides (Vonk AG *et al*., 2002). However, due to their large size, filaments are unable to be phagocytosed and thus neutrophils must find other modes of containment. To contain extracellular filaments, neutrophils produce extracellular traps (termed NETs) in which they spew extracellular fibers consisting of chromatin and cytoplasmic proteins onto the filaments, effectively damaging and killing the fungus (Urban CF *et al*., 2005, Fuchs TA *et al*., 2007, Hopke A *et al*., 2016). Occasionally, intracellular germination is induced *in vitro* by yeast cells inside neutrophils, and this may be caused by defects in myeloperoxidase production, which is an important antimicrobial enzyme found inside the phagolysosome. In these dysfunctional neutrophils, less damage occurs to filaments, suggesting that this enzyme is necessary for response to yeast

and inhibition of intra-phagolysosomal germination (Diamond RD *et al*., 1980). As another first responder, macrophages can recognize and ingest yeast cells; however, cell wall differences in filaments render the morphotype relatively unrecognizable by macrophages (Gantner BN *et al*., 2005). Surprisingly, macrophages are unable to control germination of yeast cells *in vitro*, and filaments are able to burst through these cells and kill them, although the mechanism behind this lack of control remains unknown (Calderone RA and Clancy CJ, 2012). The antigen-presenting dendritic cells are also important during innate recognition of *C. albicans* infection and are necessary for initiating adaptive immunity. Dendritic cells mediate recognition of yeast and filaments through similar receptors/pathways as macrophages and neutrophils. Further, these cells are successful at engulfing both yeast and filaments, and process each for differential T-cell activation (d'Ostiani CF *et al*., 2000). Overall, these studies suggest that neutrophils, macrophages, and dendritic cells all have functions that are important for containment, antigenic presentation, damage, and killing of specific *C. albicans* morphotypes.

1.4 Important and Relevant Infection Models

In vitro cell lines and *in vivo* animal models are used to study the host-pathogen interactions that occur during *C. albicans* infections, and to understand the basic functions of the immune system and fungal infection mechanisms. Both *in vitro* and *in vivo* models provide powerful platforms to understand how specific host cells interact with the fungus, and how the progression of infection occurs in a live model.

1.4.1 *In Vitro* Model Systems

In vitro cell lines have provided significant feedback on how yeast and filaments interact with specific immune cells and host epithelial and endothelial barriers. *C. albicans* is thought to invade both epithelial and endothelial tissues during invasive infections in humans (Grubb SEW *et al*., 2008). Thus, both epithelial and endothelial monolayer cell lines and reconstituted tissues have been utilized to analyze this aspect of infection. *In vitro* data suggests that filaments can invade these host barrier tissues via two separate mechanisms: either actively penetrating

through the host cell or via host cell-induced endocytosis (Grubb SEW *et al*., 2008). In contrast, yeast cells have not been shown to invade these tissues through either mechanism, and are only thought to initiate adherence to host tissues (Dalle F *et al*., 2009). Infections of both oral epithelial and gut epithelial cell lines result in filament invasion of both cell types (Dalle F *et al*., 2009, Zhu W and Filler SG, 2010). Filaments also invade endothelial cells via active penetration and induced endocytosis, and cause damage that has been quantified with chromium release and lactate dehydrogenase assays (Filler SG *et al*., 1995, Wachtler B *et al*., 2011). In both reconstituted human vaginal epithelium and reconstituted human oral epithelium models, filament production is necessary for the initiation of invasion, as hypofilamentous strains provoke significantly less damage and inflammatory response compared to wildtype or hyperfilamentous infections (Moyes DL *et al*., 2011, Peters BM *et al*., 2014, Jayatilake JAMS *et al*., 2006). Thus, *in vitro* models of epithelial and endothelial cells have provided major insight into the role of filaments in tissue invasion and damage.

Phagocyte function in relation to infection has also been studied using *in vitro* neutrophil and macrophage cell lines. Both yeast and filaments can be recognized by neutrophils, resulting in phagocytosis and extracellular trap formation *in vitro* (Hopke A *et al*., 2016, Urban CF *et al*., 2006). Further, neutrophils deficient in immune signaling or killing components, such as CARD9 or myeloperoxidase, are unable to control germination of the fungus, suggesting that intact immune response and subsequent signaling cascades are important in control of the fungus, especially during germination (Drewniak A *et al*., 2013, Diamond RD *et al*., 1980). Murine macrophages are also able to phagocytose fungal strains deficient in adhesins and filamentous growth, presumably due to their yeast-like growth (McKenzie CGJ, *et al*., 2010). However, intracellular filamentous growth is necessary for the induction of inflammasome and proinflammatory cytokine expression by macrophages, as the yeast-locked *efg1*Δ/Δ */cph1*Δ/Δ mutant results in diminished IL-1β expression (Joly S *et al*., 2009). Thus, *in vitro* phagocyte models have provided significant evidence of yeast and filament recognition and phagocytosis,

the importance of morphotype for immune activation, and the importance of intact immune response components such as antimicrobial molecules.

Although important information on host-pathogen interactions has been acquired *in vitro,* it is important to note that these cell lines do not completely capture the intact host environment. The host barrier models are important for demonstrating filament roles during invasion and damage to host tissues, but disseminated infection cannot be visualized without an intact host and blood flow. Not all host protein and immune components are present during *in vitro* infections as well, which may alter how individual immune cells and epithelial/endothelial cells function. Further, although most of these cell lines originate from humans or mice, these models still do not always recapitulate what occurs *in vivo*. Thus, intact *in vivo* model systems are necessary to capture similarities in human infections and more precise host-pathogen interactions.

1.4.2 *In Vivo* Model Systems

In vitro models capture many aspects of infection, but do not always recapitulate what occurs in intact hosts. Therefore, *in vivo* models, such as the mouse and the zebrafish, must be utilized to understand the host-pathogen interactions that occur in a host with physical and biological components that are not always present *in vitro*. These interactions can more closely replicate what occurs in human infection, and parallels in infection progression and immune response can be analyzed across species.

1.4.2.1 *Mus musculus*: The Mouse

The most well-known and utilized *in vivo* model for candidiasis infections is the mouse. While small, mice have anatomical and immunological components similar to humans, and genetic manipulation allows for investigation of specific genes necessary for immune function during fungal infection (Naglik JR *et al*., 2008). Both oropharyngeal and vaginitis mucosal models recapitulate human infection fairly closely, and hematogeneous infections have provided major insight into the repercussions of invasive infections (Rahman D *et al*., 2007, Papadimitriou

JM and Ashman RB, 1986). However, mice must be immunosuppressed through steroid or antibiotic treatment for colonization and infection to ensue in these models, as *C. albicans* is not a natural colonizer of mice (Naglik JR *et al*., 2008, Jawhara S *et al*., 2008). Further, the opacity of mice does not allow for real-time visualization of infections, and requires endpoint assays to determine infection status. Regardless of these limitations, mice have provided significant assessments of *C. albicans* mechanisms of infection.

Multiple mucosal models have been established in the mouse. Vaginitis infections are usually dependent on continuous infusion of estrogen due to the short reproductive cycle in mice. However, this model can be colonized for a long period of time (5-6 weeks) and is similar to human vaginal colonization (Naglik JR *et al*., 2008). Studies have shown that yeast-locked infections, such as those with *efg1*Δ/Δ /*cph1*Δ/Δ or the overexpression *NRG1* strains, result in diminished invasion of tissue and reduced inflammatory responses, whereas wildtype infections germinate, causing tissue invasion and damage (Peters BM *et al*., 2014, Fulurija A *et al*., 1996). During oral infection of mice, classical symptoms of thrush occur. These studies have shown that filaments invade epithelial tissues and cause major damage, leading to an influx of inflammatory cytokines and phagocytes (Takakura N *et al*., 2003, Hise AG *et al*., 2009). Further, a model of gastrointestinal tract colonization and systemic infection has been established using immunosuppression. In this model, a constitutively filament-dominant strain (*tup1*Δ/Δ knockout) colonizes the GI tract significantly less than a wildtype or yeast-locked mutant (*efg1*Δ/Δ */cph1*Δ/Δ double knockout), and also displays no mucosal disruption or disseminated infection, suggesting that presence of the yeast form may be important for infection (Koh AY *et al*., 2008). Overall, these murine models have provided feedback on the importance of morphogenesis and inflammatory responses during mucosal infections *in vivo*.

Multiple systemic models have also been established in the mouse. Systemic candidiasis infections usually occur through two routes, tail vein injections directly into the bloodstream, and intraperitoneally (Young G, 1958, Spellberg B *et al*., 2005). Each of these infections causes

either bloodstream-to-tissue (bloodstream) dissemination or tissue-to-bloodstream (peritoneum) dissemination. During both types of infections, yeast cells somehow escape from the bloodstream or peritoneum and travel to multiple organs, most notably to the kidneys where histological samples have shown major tissue damage from filamentous invasion (Saville SP *et al*., 2003, Carlisle PL *et al*., 2009). Other organs, such as the brains and livers, can also be colonized by yeast and filaments during systemic infections, and mice usually succumb to these infections (Lionakis MC *et al*., 2011, Lee SJ *et al*., 2003). Further, in another intraperitoneal study, germination and hydrolytic enzyme production were both implicated in, and necessary for, dissemination from the peritoneum and damage to organs (Kretschmar M *et al*., 1999). Thus, these models demonstrate important aspects for both bloodstream-to-tissue and tissue-to-bloodstream infections, and the potential importance of each morphotype in dissemination and tissue invasion.

Overall, the mouse model of candidiasis has greatly contributed to the understanding of infection progression in both mucosal and systemic models. However, due to immunosuppressive and opacity limitations, insights into how morphological switching and roles of morphotypes remain largely unknown and thus other models are necessary to study.

1.4.2.2 *Danio rerio*: The Zebrafish

The zebrafish has recently become an extremely powerful model organism in the context of microbial infections. The small vertebrate has many conserved immune functions compared to both mice and humans, and develops each arm of the immune system separately. Due to its transparency, the fish allows the simultaneous longitudinal study of host-pathogen interactions intravitally, enabling researchers to ask questions that cannot be investigated *in vitro* or in murine models. Further, this model is cost-effective in terms of high embryo output and ease of genetic manipulation, rendering it a useful, tractable model (Tobin DM *et al*., 2012, Lieschke GJ and Currie PD, 2007).

1.4.2.2.1 Modeling Mucosal Candidiasis in Larval Zebrafish

A model of mucosal candidiasis has been established in the swimbladder of larval zebrafish. Fish inflate their swimbladders, which are homologous to human lungs, at 4-5 days post-fertilization (dpf), providing an easy method of infection (Gratacap RL *et al*., 2014). Studies show that during wildtype infections, *C. albicans* grows dimorphically and induces proinflammatory NF-κB activity in epithelial tissues surrounding the air bubble. (Gratacap RL *et al*., 2013). Further, neutrophils are recruited to the site of infection, and once they infiltrate the swimbladder, they attack filaments with NETs, effectively inhibiting their growth and invasion of epithelial tissue. The NETs are also able to damage the filaments; however, yeast cells are generally left unscathed during these attacks. Filament invasion into epithelial tissue also precedes mortality in this model (Gratacap RL *et al*., 2017). The swimbladder model has also been utilized in the study of polymicrobial infections involving *C. albicans*. For instance, infections of both *Pseudomonas aeruginosa* and *C. albicans* promote synergism amongst the pathogens and increased pathogenesis in fish. Interestingly, an increase in fungal burden in the swimbladder does not correlate with an increase in filament production, suggesting that germination is not a sole factor in the enhanced pathogenesis between the two species (Bergeron AC *et al*., 2017). Overall, modeling mucosal infection in the swimbladder of zebrafish has provided multiple insights into differentiated immune responses between morphotypes, the importance of filaments for tissue invasion, and the interactions between *C. albicans* and bacteria.

1.4.2.2.2 Modeling Systemic Candidiasis in Zebrafish

Critical to the study described in this dissertation, systemic candidiasis infections have also been implicated and studied in the larval zebrafish. The first study analyzing infections of the larval zebrafish analyzed two localized infection areas: the hindbrain ventricle and the yolk. Hindbrain infection with a wildtype strain resulted in significant germination, while yolk infections resulted in significant immune cell recruitment (Chao CC *et al*., 2010). This study also analyzed

disseminated infections in adult zebrafish using an intraperitoneal infection method. In these infections, the yeast-locked *efg1*Δ/Δ */cph1*Δ/Δ mutant resulted in attenuated virulence, but was still able to disseminate from the peritoneum into the livers of fish. However, wildtype infection caused significant mortality, and histological analyses of liver tissues found significant infiltration and damage from filaments, suggesting a role for filaments in tissue damage (Chao CC *et al*., 2010, Chen YY *et al*., 2013). More recent larval studies of the hindbrain ventricle infection site have revealed that wildtype *C. albicans* yeast escape the compartment and disseminate into the bloodstream where they can germinate in other tissues, causing high rates of mortality (Brothers KM *et al*., 2011, Brothers KM and Wheeler RT, 2012). Further, infections involving the *efg1*Δ/Δ*/cph1*Δ/Δ yeast-dominant strain result in attenuated virulence, although the strain was able to disseminate. In addition, a wildtype strain germinates in the hindbrain but grows as a yeast form once immune cells infiltrate the site of infection (Brothers KM *et al*., 2011). At the site of infection, neutrophils are able to phagocytose yeast cells, and some were also found wrapped around filaments, suggesting that neutrophils respond to both morphotypes in the zebrafish (Brothers KM *et al*., 2011). However, macrophages are unable to kill yeast after engulfment, nor return to circulation, although they prevent germination from occurring. Interestingly, in NADPH oxidase-deficient macrophages, recruitment to the site of infection and phagocytosis are diminished, allowing germination to occur prior to mortality (Brothers KM *et al*., 2011, Brothers KM *et al*., 2013). Taken together, the establishment of the systemic zebrafish infection model has been important in understanding immune responses to morphotypes and the potential importance of filaments in tissue damage and mortality. Further, this model has only begun to elicit analyses of morphotype-specific fungal infection mechanisms during disseminated disease.

Overall, the zebrafish model allows researchers to visualize the host-pathogen interactions occurring in real-time, which is unable to occur as easily, if at all, in other mammalian models. These studies provide supporting evidence of the usefulness of this model in studying host-*C.*

albicans infections, and asks questions that cannot easily be investigated in other models. Further, the establishment of a larval systemic infection model provides the groundwork for the following dissertation research involving the characterization of *C. albicans* dimorphic roles during invasive infections.

1.5 Summary

Throughout our lives, our bodies work to fight off microbial pathogens that have adapted to differentiating themselves in order to evade immune recognition and make us sick. Although viruses and bacteria receive the most attention of these infections, fungi are capable of causing deadly disease not just in humans, but in plants and animals, and are responsible for major economic and ecologic burdens throughout the world. Many of these fungal pathogens have the ability to physically alter their shape in order to promote pathogenesis in the body. This physical differentiation is thought to be a major virulence factor for infection, in particular for *Candida albicans*, which can alter its shape between a unicellular yeast form and a multicellular filament form in both mucosal and systemic infections. Invasive *C. albicans* fungal infections affect thousands of people every year, and an increase of this health problem continues to escalate due to immunodeficiencies and newly formed resistances to current antifungal drugs. Significant advances have been made in the field to understand how this pathogen interacts with the immune system, and how it can transform from a commensal to a pathogenic stage. However, many questions remain about these infections, including the exact nature of dimorphic transition *in vivo*, as well as in-depth mechanisms through which the fungus changes shape to invade tissue and spread disease. Further, a major shortcoming of prior studies in this context has been the lack of visualization of these shape transitions at high resolution in a live animal model. Thus, new infection models and techniques must be designed to investigate these yet-to-be answered questions and to eventually mediate the characterization of novel therapies.

Both *in vitro* and mouse models have been indispensable for studying host-*C. albicans* interactions; however, limitations exist within both models that restrain our ability to answer

many questions. In general, *in vitro* models have been extremely valuable in determining the role of filaments in the damage and death of host cells. Further, these models have also suggested that a differential immune response to yeast and filaments occurs during infection, proposing the idea that filaments are invasive. However, due to the monolayer structure of these cells, and the lack of an intact host, analyzing dissemination mechanisms is not feasible*. In vivo* mouse models have also highlighted the capabilities of *C. albicans* to spread disease through the bloodstream and eventual arrival into various organs for colonization and invasion, prompting the hypothesis that yeast cells might be important for disease spread in the host. However, real-time longitudinal imaging of fungal spread and invasion in this host is not possible due to opacity limitations. Thus, alternative models must be created to investigate these hypotheses.

The transparent larval zebrafish has recently become an important model in the study of both mucosal and disseminated *C. albicans* disease. Studies on disseminated and mucosal infections in zebrafish have provided powerful evidence of innate immune function against both yeast and filaments, while suggesting that filaments are important for invasion and mortality. However, the in-depth specific functions of yeast and filaments, and the role of dimorphic transition have yet to be fully examined in this model in the context of invasive, disseminated infection. Thus, this dissertation aims to investigate these roles during tissue-to-bloodstream infection to identify morphotype-function relationships and dimorphic transition importance during pathogenesis. By rigorously analyzing the roles of these shapes during infection, we can provide a better foundation for the eventual targeting of these morphotypes with novel therapeutics to inhibit their growth and the inevitable deterioration of afflicted patients.

CHAPTER 2

CHARACTERIZING THE ROLE OF FUNGAL SHAPE IN A ZEBRAFISH MODEL OF INVASIVE CANDIDIASIS

2.1 Introduction

Differentiation of shape during infection can affect how a pathogen evades the immune system and develops disease. During invasive human fungal infections, some pathogenic fungi transition between yeast and filament forms to cause detrimental disease, while others cause infections as single yeast or cyst morphotypes (Dagenais TRT and Keller NP, 2009, Frenkel JK, 1976, Medoff G *et al*., 1987, Okagaki LH *et al*., 2010, Retallack DM and Woods JP, 1999, Satoh K *et al*., 2009, Silva S *et al*., 2011). *Candida albicans* is unusual among fungal pathogens, as it grows in both yeast and filamentous form during infection of humans, mice, zebrafish and invertebrate hosts (Brothers KM *et al*., 2011, Filho AC *et al*., 2014, Cutler JE, 1991, Drummond RA *et al*., 2015, Graham CE *et al*., 2017, Samaranayake YH *et al*., 2001, Trevijano-Contador N *et al*., 2016, Vazquez-Torres A and Balish E, 1997). While both forms contribute to virulence, it is still unclear how this differentiation between shapes drives specific aspects of pathogenesis.

Candida is the most common hospital-acquired fungal infection in the United States, and the fourth most common bloodstream infection overall (Horn DL *et al*., 2009), with invasive infections causing up to 50% mortality in those affected (Gudlaugsson O *et al*., 2003). *C. albicans* can transition between yeast and filament growth, and both morphotypes have been long-hypothesized to possess specific roles for pathogenesis and disease spread in the host: yeast are presumed to spread infection while filaments are implicated in tissue damage and destruction (Gow NAR *et al*., 2011). Despite contributions from both murine and *in vitro* models in the understanding of fungal dimorphism during invasive infections, we are still held back by an inability to completely visualize morphotype-function relationships in the host.

C. albicans regulates growth between yeast and hyphal form by integrating many environmental cues, including temperature, pH, oxygen, carbon dioxide and nutrients (Sudbery

PE *et al*., 2004, Shapiro RS *et al*., 2009, Kadosh D, 2017). Several genetic pathways transduce these shifts and other environmental signals to positively and negatively regulate morphological switching through filament-specification genes such as *CPH1*, *EFG1*, *EED1* and *UME6*, and yeast-promoting regulators such as *TUP1* and *NRG1* (Han TL *et al*., 2011). Overexpression of negative regulators like *NRG1* confers a hypofilamentous phenotype, whereas overexpression of filament-specific genes like *UME6* result in hyperfilamentous growth *in vivo* (Peters BM *et al*., 2014, Saville SP *et al*., 2003).

The importance of *C. albicans* morphological transitions has made them a target for therapeutic exploration. Recent discoveries of small molecule inhibitors have shown that filamentous growth can be blocked both *in vitro* and *in vivo*, with little to no detrimental effect on *in vitro* cell lines or animal models (Graham CE *et al*., 2017, Romo JA *et al*., 2017, Vila T *et al*., 2017). Now that we have the prospect of regulating morphogenesis without killing the fungi, it is a critical time to determine the roles of each morphotype in driving both spread and invasion during infection.

Early in the study of *C. albicans* as a pathogen, deductive reasoning led to the hypotheses that filaments are more likely to be invasive and yeast have a smaller shape that can more readily spread throughout the host (Cutler JE, 1991, Vazquez-Torres A and Balish E, 1997). In fact, infections with hypofilamentous mutant strains of *C. albicans* lead to rapid clearance and low mortality, but leave dissemination relatively intact (Lo HJ *et al*., 1997, Saville SP *et al*., 2003). Some hyperfilamentous strains are also reduced in virulence compared to wildtype, even though they can still cause tissue damage and some mortality (Carlisle PL *et al*., 2009, MacCallum DM and Odds FC, 2005, Clearly IA *et al*., 2012, Shen J *et al*., 2008, Uppuluri P *et al*., 2012). Complementary to these studies, *in vitro* infections of epithelial and endothelial tissues have shown that filaments are required for epithelial invasion and damage (Dalle F *et al*., 2009, Filler SG *et al*., 1995). These results are consistent with the original hypothesis that yeast are better suited for disease spread than damaging invasion, whereas filaments are less

involved in spread but important for invasion, virulence, and infection progression. However, the attenuated virulence found in both yeast-locked and filament-locked infections suggests that the combined activity of yeast and filaments is important for pathogenesis.

Limitations remain for both *in vitro* and murine models in testing the roles of shape in *C. albicans* pathogenesis. For instance, dissemination cannot be dissected *in vitro* or in invertebrate models due to the lack of vertebrate host anatomy (Phan QT *et al*., 2000, Pukkila-Worley R *et al*., 2009, Arvantis M *et al*., 2013). Moreover, the size and opacity of mice prohibits the tracking of individual fungal cells and morphotype differentiation during infection progression (Jacobsen ID *et al*., 2014, Mitra S *et al*., 2010, Shi M and Mody CH, 2016, Vanherp L *et al*., 2018). Despite hypotheses correlating morphotype-function relationships, it has never been feasible to comprehensively test the relative abilities of individual filament and yeast cells during invasion or spread to the bloodstream from tissue during *in vivo* infection. To investigate these hypotheses with intravital imaging, and bridge the gaps between *in vitro* and murine work, we sought to adapt the larval zebrafish as a model to examine fungal and host determinants of pathogenesis.

The larval zebrafish offers a unique vertebrate model for studying bacterial, viral, and fungal infections (Goody MF *et al*., 2014, Gratacap RL and Wheeler RT, 2014, Neely MN *et al*., 2002). This transparent host allows the simultaneous longitudinal study of host-pathogen interactions intravitally, enabling us to ask questions we cannot investigate *in vitro* or in murine models. Non-invasive imaging enables us to monitor the location and shape of all fungal cells during infection and measure the specific morphotype-function relationships of yeast and filaments. Further, the environmental resiliency of zebrafish allows us to alter infection temperature *in vivo*, augmenting our ability to manipulate fungal morphology in concert with conventional genetic manipulation.

Here, we describe a new zebrafish yolk infection model and leverage this infection route to study tissue-to-bloodstream dissemination of *C. albicans*. Multiple genetic and environmental perturbations were used to morphologically bias the population and test the roles of each

morphotype during disease development. Our results confirm the specialized functions of yeast in infection spread and filaments in tissue invasion. Further, our data suggest that each shape has an independent, additive effect on dissemination and mortality that is not enhanced by the presence of other morphotypes. Thus, by using orthologous tools to manipulate the fungus, in combination with whole animal imaging, we demonstrate the consequences of *C. albicans* morphological diversity during disease development in the host.

2.2 Materials and Methods

2.2.1 Zebrafish Care and Maintenance

Adult zebrafish were kept at 28°C in recirculating systems (Aquatic Habitats, Apopka, FL) at the University of Maine Zebrafish Facility. Zebrafish embryos were collected and grown at 33°C at a density of 150 eggs/dish in 150 mm petri dishes containing 150 mL egg water (nanopure water with 20 g/L [60 mg/L final concentration] Instant Ocean salts [Aquarium Systems, Mentor, OH]). Egg water was supplemented with 0.1% methylene blue (final concentration 0.00003%) (Alfa Aesar, Haverhill, MA) for 24 hours to prevent microbial growth. Water was changed once a day to keep larvae clean. For temperature experiments, larvae were placed at either 33°C, 28°C, or 21°C after microinjection. Wildtype AB from the Zebrafish International Resource Center and *Tg(BACmpo:gfp)* (marks neutrophils) as described (Renshaw SA *et al*., 2006) fish lines were used for experiments. All zebrafish husbandry was performed as described previously (Nüsslein-Volhard C and Dahm R, 2002, Westerfield M, 2000).

2.2.2 Ethics Statement

All zebrafish protocols were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council (NRC, 2011). All animals were treated in a humane manner according to the guidelines of the University of Maine Institutional Animal Care and Use Committee (IACUC) protocols A2012-11-03 and A2015-11-03.

2.2.3 Fungal Strains and Growth Conditions

Candida albicans strains are described in Table 2.1. The non-fluorescent overexpression strains were described previously (Peters BM *et al*., 2014). *C. albicans* strains were grown on YPD agar (yeast-peptone-dextrose) (BD/Fisher Chemical: 20 g/L Bacto Peptone, 10 g/L Bacto Yeast Extract, 20 g/L Bacto Agar, 20 g/L dextrose) at 37°C. Liquid cultures were grown overnight in YPD at 30°C for infections. For UME6^{OEX} infections, strain was grown on YPD agar or liquid supplemented with, respectively, 50 µg/mL or 25 µg/mL doxycycline hyclate (Calbiochem, EMD Millipore, Billerica, MA) to produce non-filamentous colonies. Overnight cultures were diluted or resuspended in phosphate-buffered saline (PBS) and counted on a hemocytometer for a concentration of $1x10^7$ cells/mL or $5x10^6$ cells/mL for injection. For mixed infections, concentrations of each fungal strain ($1x10^7$ cells/mL) were made separately before mixing together in a 1:1 ratio for injection (final concentration for each strain at $5x10^6$ cells/mL). For infections involving UME6^{OEX}-dTomato, yeast cells were stained for 5 minutes with 750 µg/mL calcofluor white (CFW, Sigma-Aldrich, St. Louis, MO) before washed in PBS 1-2x for better visualization during post-infection screening.

Strain	Parent Strain	Genotype	Reference
SC5314-GFP		Wildtype clinical isolate, pENO1-EGFP-NAT^R	Hopke A *et al*., 2016
***efg1* Δ/Δ *cph1* Δ/Δ-dTomato**	CAI4	*ura3*::*1 imm434*/*ura3*::*1 imm434 cph1*::*hisG*/*cph1*::*hisG efg1*::*hisG*/*efg1*::*hisG*-URA3-*hisG* pENO1-dTomato-NAT^R	This study, Brothers KM *et al.,* 2011
***eed1* Δ/Δ-dTomato**	CAI4	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 edt1*Δ::*hisG edt1*::*hisG*::URA3 pENO1-dTomato-NAT^R	Brothers KM *et al*., 2013
TT21-dTomato	THE21	*ade2*::*hisG*/*ade2*::*hisG ura3*::*imm434*/*ura3*::*imm434*::URA3 tetO-ENO1/*eno1*::ENO1 tetR-ScHAP4AD-3XHA-ADE2 pENO1-dTomato-NAT^R	This study, Peters B *et al.*, 2014
NRG1^{OEX}-dTomato	THE21	*ade2*::*hisG*/*ade2*::*hisG ura3*::*imm434*/*ura3*::*imm434*::URA3-tet-O-NRG1 ENO1/*eno1*::ENO1 tetR-ScHAP4AD-3XHA-ADE2 pENO1-dTomato-NAT^R	This study, Peters B *et al.*, 2014
NRG1^{OEX}-NEON	THE21	*ade2*::*hisG*/*ade2*::*hisG ura3*::*imm434*/*ura3*::*imm434*::URA3-tet-O-NRG1 ENO1/*eno1*::ENO1 tetR-ScHAP4AD-3XHA-ADE2 pENO1-NEON-NAT^R	This study, Peters B *et al.*, 2014
UME6^{OEX}-dTomato	THE21	*ade2*::*hisG*/*ade2*::*hisG ura3*::*imm434*/*ura3*::*imm434*::URA3-tet-O-UME6 ENO1/*eno1*::ENO1 tetR-ScHAP4AD-3XHA-ADE2 pENO1-dTomato-NAT^R	This study, Peters B *et al.*, 2014
NRG1^{OEX}-iRFP	THE21	*ade2*::*hisG*/*ade2*::*hisG ura3*::*imm434*/*ura3*::*imm434*::URA3-tet-O-UME6 ENO1/*eno1*::ENO1 tetR-ScHAP4AD-3XHA-ADE2 pENO1-iRFP-NAT^R	This study, Bergeron AC *et al*., 2017, Peters B *et al.*, 2014

Table 2.1 Strains Used in this Study.

2.2.4 Engineering of dTomato, iRFP, and NEON-expressing *C. albicans* Strains

The NRG1^{OEX}-dTomato-NAT^R, TT21-dTomato-NAT^R, and UME6^{OEX}-dTomato-NAT^R *C. albicans* strains were constructed by transforming NRG1^{OEX}, TT21, and UME6^{OEX} strains (Table 2.1, Peters BM *et al*., 2014) with the pENO1-dTomato-NAT^R plasmid engineered in our lab. The NRG1^{OEX}-iRFP-NAT^R *C. albicans* strain was constructed by transforming the NRG1^{OEX} strain with the pENO1-iRFP-NAT^R plasmid engineered in our lab (Table 2.1). The NRG1^{OEX}-NEON-NAT^R *C. albicans* strain (NRG1^{OEX}-NEON) was constructed by transforming the NRG1^{OEX} strain (Table 2.1) with the pENO1-NEON-NAT^R plasmid engineered in our lab. These plasmids contain a codon-optimized version of the dTomato, iRFP, or NEON gene under the control of the constitutive *ENO1* promoter, with a nourseothricin resistance selection marker (NAT^R) (plasmids themselves are on a pUC57 backbone). Plasmids were cloned and ligated into *E. coli* competent strain NEB5α (New England BioLabs, Ipswich, MA). Transformed cells were miniprepped (QIAprep Spin Miniprep Kit, Qiagen, Valencia, CA) and DNA was digested with

restriction enzymes flanking the beginning and end of the dTomato, iRFP, or NEON inserts (NcoI and PacI, New England BioLabs, Ipswich, MA) to identify correct transformants (backbone is ~5 kb, dTomato insert is 702 bp, iRFP insert is 940 bp, NEON insert is 715 bp). *C. albicans* was transformed using the lithium acetate protocol previously published (Gietz RD *et al*., 1995), with nourseothricin resistance as a selection marker (100 µg/mL NAT, Werner Bioagents, Jena, Germany). At least 3-20 colonies were selected and screened for fluorescence via epifluorescence microscopy (Carl Zeiss Microscopy LLC, Thornwood, NY) and flow cytometry (dTomato: 558 nm laser with DsRed filter, iRFP: 640 nm laser with APC filter, NEON: 488 nm laser with FITC filter, LSRII, Becton, Dickinson and Company, Franklin Lakes, NJ). PCR was used to check for correct integration of the dTomato plasmid using primers Sp6 and dTomato Rv (~680 bp) or P_{ENO1} Fw and dTomato Rv (~1.2 kb) (Gratacrap RL *et al*., 2013). PCR was used to check for correct integration of the iRFP plasmid using primers P_{ENO1} Fw and iRFP Rv1 (~1.2 kb) (Bergeron AC *et al*., 2017). PCR was used to check for correct integration of the NEON plasmid using primers P_{ENO1} Fw and NEON Rv (~1.2 kb). All primer sets can be found in Table 2.2.

Primer Name	Sequence	Reference
P_{ENO1} Fw	5'-TCCTTGGCTGGCACTGAACTCG-3'	Gratacap R *et al*., 2013
Sp6 universal	5'-GATTTAGGTGACACTATAG-3'	Brown JE *et al*., 1986
dTomato Rv	5'-AAGGTCTACCTTCACCTTCACC-3'	Gratacap R *et al*., 2013
NEON Rv	5'-CATGAGTAGCTGGCAATGAAGC-3'	This study
iRFP Rv1	5'-ATCACATGAAGTCAAATCAACTTTTCTAGC-3'	Bergeron AC *et al*., 2017

Table 2.2 PCR Primers Used in this Study.

2.2.5 Microinjection

Zebrafish at the roughly prim-22 stage were physically dechorionated and anesthetized in 4 mg/mL Tris-buffered tricaine methanesulfonate (final concentration 160 µg/mL in 50 mL egg water) (Western Chemical, Ferndale, WA). For injection, 2-4 nL of PBS or *C. albicans* suspended at $1x10^7$ cells/mL or $5x10^6$ cells/mL in PBS was microinjected with borosilicate glass capillary needles (Sutter Instrument, Novata, CA) through the back of the yolk near the junction of the yolk extension for a dose between 5-20 CFUs. For injections needing doxycycline (DOX),

250 μg/mL DOX was injected alongside the *C. albicans* inoculum. In experiments that needed exact known inoculum sizes, fish were screened and inoculum sizes for all strains were counted. Fish were then plated in individual wells until imaging and infection scoring. A small portion of fish mock-infected with PBS do die due to natural causes after the first day of injection.

2.2.6 Fluorescence Microscopy

An FV-1000 laser scanning confocal system on an Olympus IX-81 inverted microscope was used for confocal imaging (Olympus, Waltham, MA). Objective lenses with powers of 10x/0.40 NA and 20x/0.70 NA were used. Live fish were prepared for imaging by anesthetizing and immobilizing them in 0.4% low melting-point agarose (Lonza, Rockland, ME) with tricaine. Intact fish images are overlays of differential interference contrast (DIC) and fluorescence images or fluorescence image panels (*e.g.*, red, red/green). Images consist of 18-25 slices stacked at a maximum Z-projection. dTomato, enhanced green fluorescence protein (EGFP), NEON, and iRFP fluorescent proteins were detected by optical lasers/filters with a 10x (numeric aperture NA, 0.4) or 20x (NA 0.7) for excitation/emission at 546 nm/560 to 660 nm (dTomato), 488 nm/505 to 525 nm (both EGFP and NEON), and 647 nm/655 to 755 nm (iRFP). For 2D imaging of yeast and filaments, fish were euthanized by overdose with tricaine (300 μg/mL for 10 minutes) and individually placed on a glass slide (25 x 75 mm, Corning, Corning, NY). A glass coverslip (12 x 12 mm, Corning, Corning, NY) was placed on top of fish and pressed down using the bulb of a plastic Pasteur pipette to gently flatten fish. Images were acquired on a Zeiss Axiovision epifluorescence microscope (Carl Zeiss Microscopy LLC, Thornwood, NY) using a 40x objective (NA 0.75) and processed using Photoshop (version CS5 12.1 x64, Adobe Systems Incorporated) and FIJI (www.fiji.sc) software.

2.2.7 Fungal Burden Assay for Colony-Forming-Units (CFUs)

Larvae infected with *C. albicans* yeast-specific NRG1^{OEX} strain were incubated at 21°C, 28°C, and 33°C for 18-24 hours. At each time point, 5 living larvae were euthanized in tricaine,

placed in 100 µL 1x PBS and homogenized with a hand-held Kontes microgrinder (Research Products International Inc., Mount Prospect, IL). 500 µL of 1x PBS was then added to the original homogenate for a total of 600 µL PBS and homogenate. Ten-fold serial dilutions were made in 1x PBS and 100 µL was plated in duplicate onto YPD supplemented with 30 µg/mL gentamicin sulfate (Lonza, Walkersville, MD), 250 µg/mL penicillin/streptomycin (Lonza, Walkersville, MD), and 3 µg/mL vancomycin hydrochloride (Amresco Inc., Solon, OH) and grown overnight at 30°C. Viable colonies were counted and CFUs were calculated per fish.

2.2.8 Imaging and Quantification of Filament Invasion and Yeast Colonization in Intact Fish

To image fungal invasion and colonization in intact fish, known yeast and/or filament inocula infected fish were plated into individual wells in either 24- or 96-well glass bottom plates, anesthetized with tricaine, and stabilized in 0.4% low-melting agarose as described above. Each fish was placed on its left side, with head facing the right side of the well. Random, non-disseminated fish were imaged at 18 hpi. Yolk sacs were imaged at 10x+1.4x zoom on an Olympus IX-81 inverted confocal microscope with consistent PMT voltage (HV: for Alexa Fluor 488 laser, 660V, for Alexa Fluor 546 laser, 700V). Images consist of 18-20 slices stacked at a maximum Z-projection. Images were then transferred to ImageJ (FIJI), a fluorescence threshold for either bright filaments (indicating invasion outside of yolk) or whole yeast colonizing areas was chosen, and area was quantified in pixels. If fish had infection on the far side of the yolk and thresholding could not detect fluorescence, these fish were excluded from data analysis. Colonizing yeast level was calculated as a percentage of the total area covered by fungi. Care was taken to ensure that images for quantification were taken with identical settings and processed identically.

2.2.9 Percent Survival Quantification

To quantify percent survival of infected fish for Kaplan-Meier curves, all fish were pooled at each time point and censored for either survival (0) or death (1) for each group (Uninfected,

Infected). Fish survival analysis was the same for all experiments, regardless of unknown or known inocula status per individual fish.

2.2.10 Percent Dissemination Quantification

To quantify percent dissemination in experiments with generally pooled infection groups (unknown inocula per individual fish: Figures 2.3B, 2.3D, 2.6A, 2.7A, 2.7C, 2.11C, 2.12C, 2.13C, 2.15B, 2.16B, 2.17A, 2.17C), all scored, live fish were pooled from each experiment at each time point. Percent dissemination was quantified by calculating the pooled number of disseminated fish divided by total number of live fish. To quantify percent dissemination in experiments where early yeast levels or filament invasion was imaged in fish with known infection inocula (18 hpi), only live fish that had been imaged were scored for dissemination and pooled from each experiment at each time point (known inocula per individual fish: Figures 2.5A, 2.19A). Individual fish with disseminated infection at both 24 and 30 hpi were analyzed at both time points. Individual live fish with disseminated infection at 24, but not 30 hpi, were analyzed as non-disseminated at 30 hpi. Percent dissemination was quantified by calculating the pooled number of imaged and disseminated fish, per time point, and divided by the total number of live fish that had been imaged.

2.2.11 Filament Invasion Analyses

Filament invasion analyses involving comparisons to filament inoculum size (Figure 2.4B), mortality (Figure 2.4C), dissemination (Figure 2.19C), and yeast inoculum size (Figure 2.20B) were analyzed using individual known inocula-infected fish that were imaged at 18 hpi for invasion. Filament and yeast inoculum comparisons to invasion were quantified by plotting individual fish with filament invasion with low (1-10 cells) yeast or filament inoculum size, and individual fish with filament invasion with high (11-20 cells) yeast or filament inoculum size. Filament invasion and mortality comparisons were quantified by plotting individual fish with filament invasion with early (24 hpi) or late (30 hpi) death. Filament invasion and dissemination

comparisons were quantified by plotting individual live fish with filament invasion with dissemination at only 24 hpi, dissemination at only 30 hpi, or that had no dissemination by 30 hpi.

2.2.12 Yeast Yolk Level Analyses

Yeast yolk level analyses involving comparisons to filament inoculum size (Figure 2.19B) and dissemination (Figure 2.5B) were analyzed using individual known inocula-infected fish that were imaged at 18 hpi for yeast levels in the yolk. Filament inoculum comparisons to yeast yolk levels were quantified by plotting individual fish with yeast yolk levels with low (1-10 cells) filament inoculum size, and individual fish with yeast yolk levels with high (11-20) filament inoculum size. Dissemination comparisons involving low (1-10) and high (11-20) yeast inoculum sizes to yeast yolk levels were quantified by plotting individual live fish with yeast yolk levels with dissemination at 24 hpi, dissemination at 30 hpi, or that had no dissemination by 30 hpi. These groups were split to include low and high yeast inoculum sizes.

2.2.13 Neutrophil Quantification of Whole Fish Using Flow Cytometry

Larval *Tg(BACmpo:gfp)* zebrafish were grown at 33°C until yolk-infected with NRG1^{OEX} or PBS at ~35 hpf. Infected and mock fish groups were then separated into three temperature groups and incubated at 21°C, 28°C or 33°C for 24 hours. At 24 hpi, fish from each group were euthanized with tricaine and homogenized on a 40 µm cell strainer (Falcon/Corning, Corning, NY) with a plastic transfer pipette (Fisher Scientific, Hampton, NH) into cold PBS/1% bovine serum albumin (BSA at 10 mg/mL, Millipore Corp, Kankakee, IL) (n=20-30 per group, per temperature). Fish homogenates were then centrifuged in a swinging bucket Sorvall Legend RT (ThermoFisher Scientific, Asheville, NC) at 1500 rpm for 5 minutes and resuspended in cold PBS/1% BSA/5 µM Sytox Orange (to gate for Live Singlets, ThermoFisher Scientific, Asheville, NC). Fish homogenates were then analyzed on an LSRII flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ) and gated at Live Singlets (Sytox Orange using dsRed channel, excitation/emission of 583nm). Neutrophils were quantified using a FITC channel at

excitation/emission of 490 nm/525 nm. Neutrophil raw numbers were divided by total events and multiplied by 100 for percent neutrophils. Four experiments were analyzed.

2.2.14 Neutrophil Quantification of Tail Fin Transection Using Sudan Black Staining

Larval AB zebrafish were grown in egg water containing N-Phenylthiourea (PTU at 27 μg/mL, Sigma-Aldrich, St. Louis, MO) at 33°C to prevent melanin production. At ~35 hpf, fish were anesthetized with tricaine, microinjected with PBS, and split into uncut (UC) and cut (C) groups (6 groups total, 3 UC for 21°C, 28°C and 33°C incubation, 3 C for 21°C, 28°C and 33°C incubation, n=25 fish per group, per temperature). Fish were then incubated at their respective temperatures for 24 hours. At 24 hpi, to-be transected fish groups (cut) were anesthetized in tricaine and tail fins were transected using a sterile razor blade on a glass slide. After transection, UC and C fish groups were then incubated for 1 hour at their respective temperatures. At 1 hpt (hour post-transection), all fish groups (n=15 per group per temperature) were euthanized with tricaine and fixed in 4% paraformaldehyde (diluted in PBS from 37% formaldehyde, Fisher Scientific, Hampton, NH) overnight at 4°C. Fixed fish were then washed with 1x PBS and stained for 25 minutes with Sudan Black B (Sigma Aldrich, St. Louis, MO). After staining, fish were extensively washed in 70% ethanol/PBS (4x), 70% ethanol/PBS-Tween20 (2x), 50% ethanol/PBS-Tween20 (2x), 30% ethanol/PBS-Tween20 (2x) and PBS-Tween20 (1x) (Ethyl alcohol 200 proof Pure, Sigma Aldrich, St. Louis, MO; TWEEN 20 Detergent, EMD Millipore Corp, Billerica, MA) before storing in 80% glycerol at 4°C (EMD Chemicals Inc, Gibbstown, NJ). Neutrophils were counted at the site of transection and numbers were pooled and averaged.

2.2.15 Statistical Analysis

All statistical analysis was performed in GraphPad Prism 6 software (La Jolla, CA). All data was tested with non-parametric tests, as indicated in the corresponding figure legends. P-values are as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, n.s. not significant.

2.3 Results

2.3.1 Yeast Growth Leads to Dissemination While Filamentous Growth Leads to Invasion and Death

To visualize the functions of different *C. albicans* morphotypes during infection, we developed a zebrafish yolk infection route in which a localized tissue infection can spread to the bloodstream. At the normal temperature for zebrafish husbandry, 28°C, wildtype *C. albicans* yeast rapidly germinate unless contained by phagocytes (Brothers KM *et al*., 2011, Gratacap RL *et al*., 2014). Therefore, to test the roles of different morphological forms, we infected fish at this temperature with either the filament-dominant wildtype strain (SC5314-GFP, Figure 2.1A-a) or hypofilamentous strains (*efg1*Δ/Δ*cph1*Δ/Δ-dTomato or *eed1*Δ/Δ-dTomato, Figure 2.1A-b/c). Although both *efg1*Δ/Δ*cph1*Δ/Δ and *eed1*Δ/Δ were hypofilamentous during infections, pseudohyphae were still observed (Figure 2.2). The production of pseudohyphae *in vivo* is also seen in mammalian infection models (Chen CG *et al*., 2006, Riggle PJ *et al*., 1999), but did complicate the interpretation of our results. The NRG1^{OEX} strain overexpresses the *NRG1* gene, which is necessary and sufficient for yeast growth (Peters BM *et al*., 2014). In contrast to the *efg1*Δ/Δ*cph1*Δ/Δ and *eed1*Δ/Δ mutants, the NRG1^{OEX}-dTomato strain was morphologically stable and yeast-locked *in vivo,* and could be compared to its strongly filament-producing control strain, TT21-dTomato (Figure 2.1B). Use of these diverse genetic manipulations to bias fungal morphology allowed us to control for possible manipulation-specific artifacts.

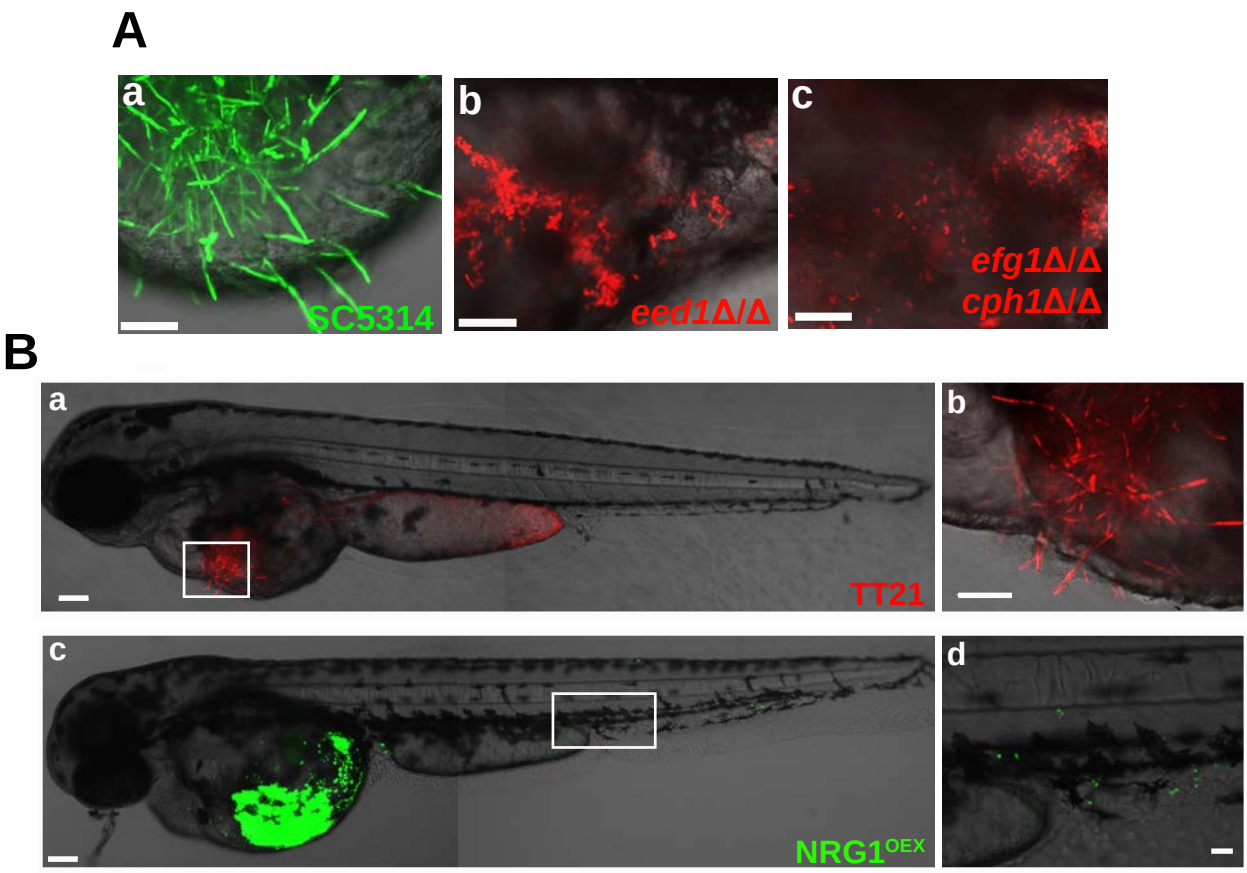

Figure 2.1: Wildtype infections result in filament growth and invasion of tissue while yeast-locked infections result in yeast growth and dissemination. Larval AB zebrafish were raised at 33°C, infected at ~35 hpf with fungi, screened for correct inoculum, and kept at 28°C post-infection. **(A)** Yolk insets of single strain infections of (a) wildtype SC5314-GFP and hypofilamentous mutants (b) *eed1*Δ/Δ-dTomato and (c) *efg1*Δ/Δ/*cph1*Δ/Δ-dTomato. **(B)** (a, c) Single strain yolk infections of TT21-dTomato and NRG1^{OEX}-NEON. (b) TT21 inset of morphology/invasion in yolk/heart. (d) NRG1^{OEX} inset of yeast disseminating in the tail. Scale bars: Panel A = 50 μm; Panel B, whole fish = 100 μm, insets = 50 μm.

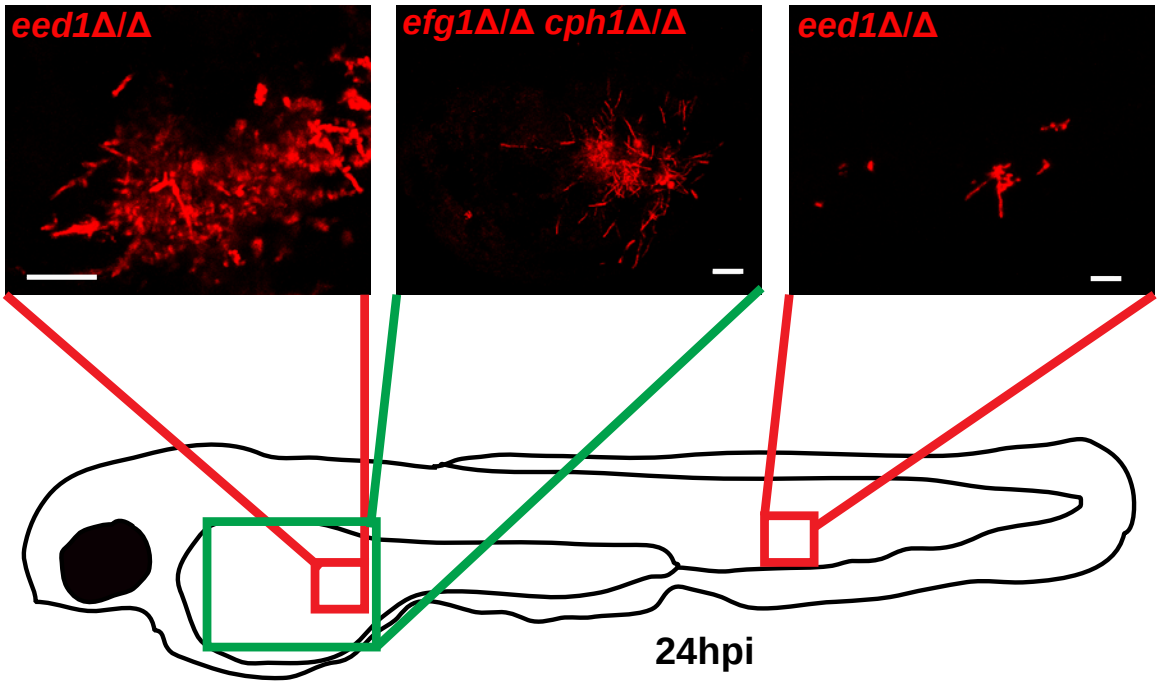

Figure 2.2: Hypofilamentous mutants grow pseudohyphae during infection. *eed1*Δ/Δ-dTomato and *efg1*Δ/Δ/*cph1*Δ/Δ-dTomato-infected zebrafish were imaged at 24 hpi for morphology. Representative images of pseudohyphal growth of the hypofilamentous mutants observed during infection in both the yolk and the tail. Drawn zebrafish schematic displays the areas of the fish where images were captured. Scale bars: 50 μm.

Filaments are found in infection foci of organs, and *in vitro* studies suggest that filaments invade and damage epithelial layers (Richardson JP *et al*., 2018). To test if filaments are highly invasive in this zebrafish infection model, and to observe the destructive power of filaments *in vivo*, we infected with filament-dominant strains SC5314 and TT21. Imaging infections by longitudinal confocal microscopy, we determined that the filament-dominant wildtype infections (SC5314 or TT21) result in very little dissemination but cause a high rate of mortality (Figure 2.3A-D). Mortality is closely associated with filaments invading the host tissue surrounding the yolk, including the heart and nearby vasculature. These experiments show that filaments are invasive, but also show that they are very ineffective at spreading through the bloodstream in the zebrafish.

Yeast-dominant mutants have been found to disseminate at higher rates from the intestinal tract, and show no loss in ability to colonize organs in an intravenous route of infection, but are hypovirulent in the murine model (Henry-Stanley MJ *et al*., 2003, Saville SP *et al*., 2003). Consistent with these previous findings, yeast-dominant infections in zebrafish with hypofilamentous mutants and the yeast-locked NRG1^{OEX} strains result in dissemination of yeast throughout the fish (Figure 2.3B, D). Despite this dissemination, the yeast are less virulent, and survival is not significantly different from uninfected controls over the first two days of infection (Figure 2.3A, C). Further, levels of both mortality/invasion from filaments and dissemination/colonization of yeast are robust over a range of infectious doses between 1-20 cells (Figures 2.4A-B, 2.5). Quantification of filament invasion in longitudinal imaging also shows that high levels of tissue invasion at 24 hpi correlate with early death of infected fish (Figure 2.4C). These results support the long-hypothesized roles of yeast and filaments during infection and validate the zebrafish as a model to study *C. albicans* morphology.

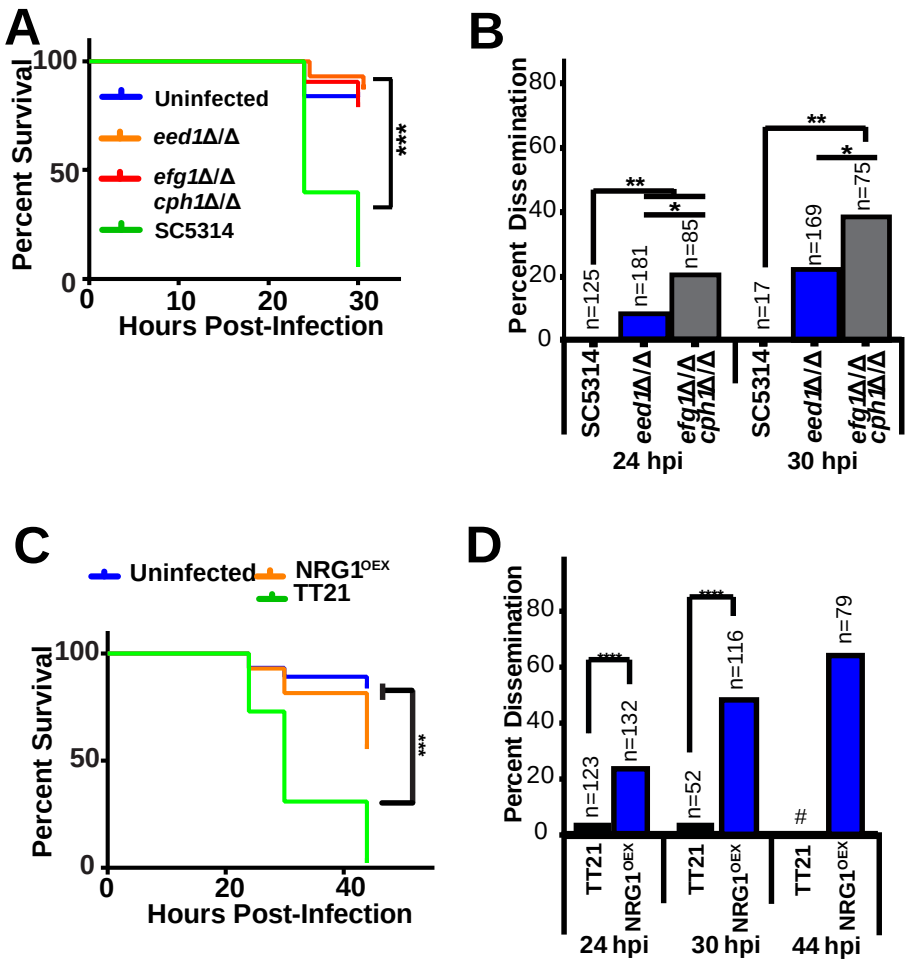

Figure 2.3: *C. albicans* filaments invade and cause death, while yeast disseminate in larval zebrafish. Larval AB zebrafish were raised at 33°C, infected at ~35 hpf with fungi, screened for correct inoculum, and kept at 28°C post-infection. Infected fish were screened and imaged on an Olympus confocal microscope at 24, 30, or 44 hpi for dissemination and mortality. **(A and B)** Infections with hypofilamentous mutants. Pooled from 9 independent experiments. **(A)** Kaplan-Meier survival curves display fish survival during infection with mutant or wildtype fungi. Pooled numbers of individual fish are the following: n=196, 194, 94, and 314 for uninfected, *eed1*Δ/Δ, *efg1*Δ/Δ/*cph1*Δ/Δ, and SC5314, respectively. **(B)** Dissemination frequencies in mutant and wildtype infections. n=number of live fish screened at time point. **(C and D)** Infections with yeast-locked NRG1^{OEX}. Pooled from 5 independent experiments. **(C)** Kaplan-Meier survival curves display fish survival during infection with wildtype (TT21-dTomato) or yeast-dominant (NRG1^{OEX}-dTomato) infections. Pooled numbers of individual fish are the following: n= 74, 142, and 168 for Uninfected, NRG1^{OEX}, and TT21, respectively. **(D)** Dissemination frequencies between wildtype and yeast-dominant infections. n=number of live fish screened at time point. Statistical analysis: (A) Mantel-Cox Log Rank test with Bonferroni correction; (B) Fisher's exact test with Bonferroni correction; (C) Mantel-Cox Log Rank test with Bonferroni correction; (D) Fisher's exact test. * p≤0.05, ** p≤0.01, *** p ≤ 0.001, **** p≤0.0001, # = data not analyzed due to more than 90% mortality.

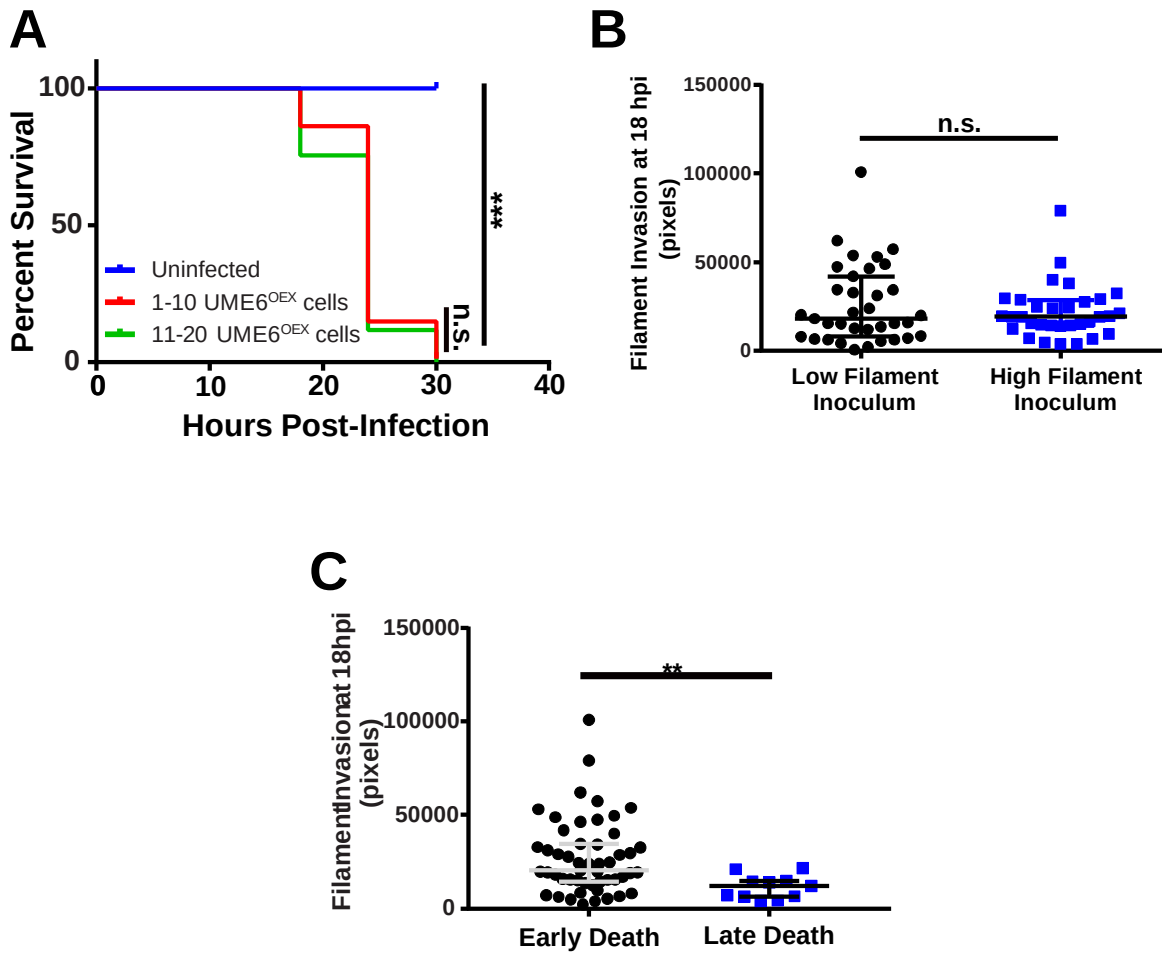

Figure 2.4: Inoculum size is not a determining factor in mortality, but invasion determines death in filament-dominant infections. Larval AB zebrafish were infected at ~35 hpf with single infections of UME6^{OEX}-dTomato, screened for exact inoculums, and individually placed in 48-well plates. Fish were kept at 28°C for the duration of the experiment. At 18 hpi, fish yolks were imaged for filament invasion and imaged fish were followed out to 24 and 30 hpi for mortality. **(A, B, and C)** Mortality and invasion in UME6^{OEX} infections with split inocula. Pooled from 4 independent experiments. **(A)** Kaplan-Meier survival curves display fish survival during infection with low (1-10) or high (11-20) UME6^{OEX} inoculums. Pooled numbers of individual fish are the following: n= 40, 195, and 85 for Uninfected, low UME6^{OEX}, and high UME6^{OEX} infections, respectively. **(B)** Filament invasion, quantified by pixels in ImageJ, in fish with either low (1-10) or high (11-20) filament inocula (black circles are low inocula, blue squares are high inocula). **(C)** Mortality in fish with filament invasion at 18 hpi. Filament invasion was quantified in confocal images taken at 18 hpi and mortality was scored at 24 and 30 hpi (fish death at 24 hpi are black circles, at 30 hpi are blue squares). Statistical analysis: (A) Mantel-Cox Log Rank test with Bonferroni correction; (B) Mann-Whitney U test, median with interquartile range displayed; (C) Mann-Whitney U test, median and interquartile range displayed. ** p≤0.01, *** p≤0.001, n.s. not significant.

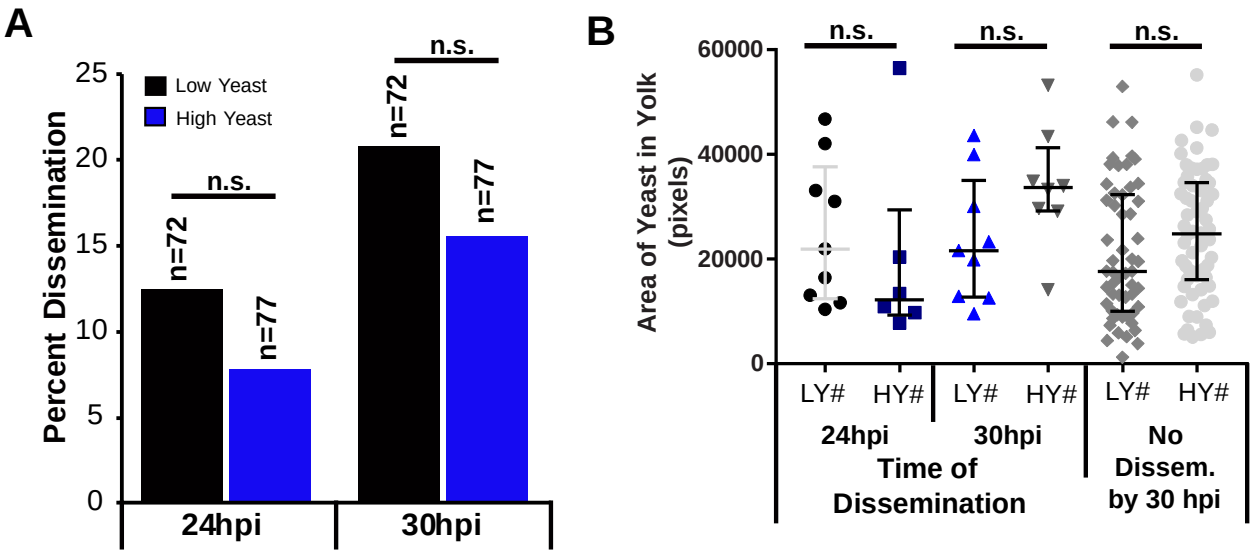

Figure 2.5: Inoculum size and yeast growth are not determining factors in disseminated yeast infections. Larval AB zebrafish were infected at ~35 hpf with single infections of NRG1^{OEX}-NEON, screened for exact inoculums, and individually placed in 48-well plates. Fish were kept at 28°C for the duration of the experiment. At 18 hpi, fish yolks were imaged for yeast growth and imaged fish were followed out to 24 and 30 hpi for disseminated infection. **(A and B)** Dissemination and yeast levels in NRG1^{OEX} infections with different yeast inocula. Pooled from 7 independent experiments. **(A)** Percent dissemination of single NRG1^{OEX} infections with low (1-10) or high (11-20) yeast inoculums. n=number of live fish screened at time point. **(B)** Yeast levels in fish with dissemination with high (HY#) or low (LY#) yeast inocula at 18 hpi. Yeast levels were quantified in confocal images taken at 18 hpi and dissemination was scored at 24 hpi or 30 hpi (black circles represent dissemination at 24 hpi with low yeast inoculum, blue squares represent dissemination at 24 hpi with high yeast inoculum, blue triangles represent dissemination at 30 hpi with low yeast inoculum, grey triangles represent dissemination at 30 hpi with high yeast inoculum, grey diamonds represent no dissemination with low yeast inoculum, and grey circles represent no dissemination with high yeast inoculum infections). Statistical analysis: (A) Fisher's exact test; (B) Mann-Whitney U test, median with interquartile range displayed. n.s. not significant.

2.3.2 Temperature Modulation of Morphology Confirms Morphotype-Function Relationship Without Affecting Zebrafish Immune Function.

While our results with hypofilamentous strains confirm the long-suspected roles of yeast and filaments in the host, the genetic modifications in these strains can affect cellular functions other than morphology (Murad AMA *et al*., 2001a, Li F and Palecek SP, 2003, Polke M *et al*., 2017, Wartenberg A *et al*., 2014, Ene IV *et al*., 2014). Zebrafish can be grown at a wide range of temperatures (Long Y *et al*., 2012 Schaefer J and Ryan A, 2006, Scott GR and Johnston IA,

2012) and yeast growth of wildtype *C. albicans* is enhanced at lower temperatures (Sudbery PE *et al*., 2004). We took advantage of these two facts and lowered the temperature during infection to enhance yeast growth *in vivo* without any mutations. We infected fish with the wildtype TT21-dTomato strain and incubated fish at the yeast-inducing 21°C. We found that the wildtype strain produces a mix of morphologies at this temperature (21.7±5.5% yeast at 30 hpi, 24.5±9.0% yeast at 44 hpi, 67.5±6.2% yeast at 68 hpi). As expected, the presence of moderate levels of yeast led to increased dissemination over time, and prolonged survival up to 68 hpi, similar to results obtained from the genetically-modified yeast-dominant strains (Figure 2.6).

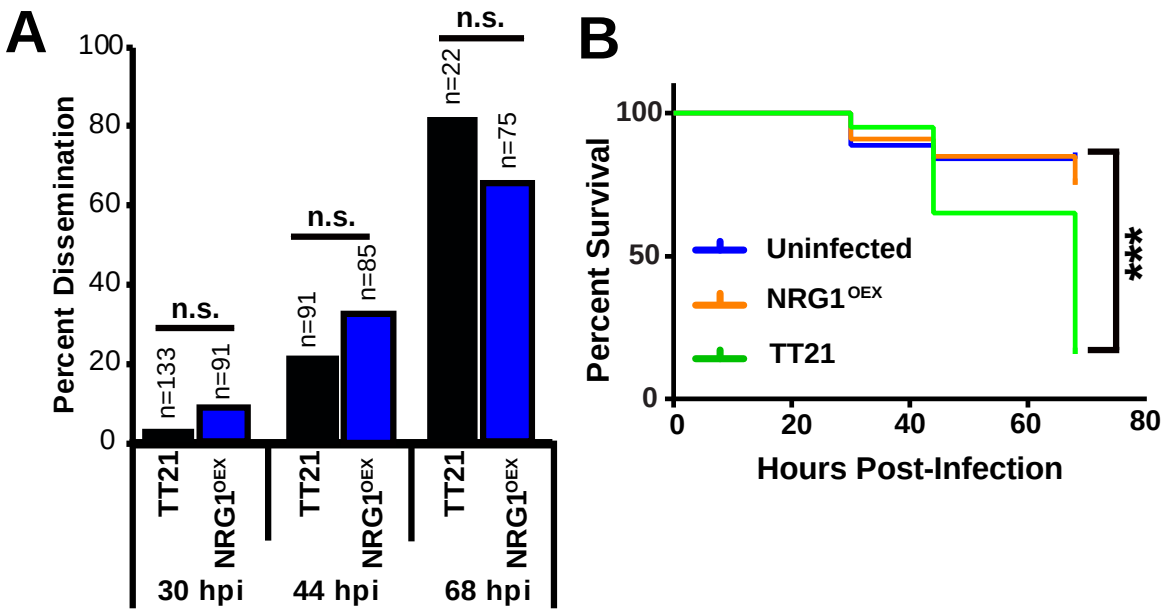

Figure 2.6: Wildtype infection can be manipulated by temperature to produce yeast, dissemination, and prolonged survival at 21°C. Larval AB zebrafish were infected at ~35 hpf with fungi, screened and incubated at 21°C post-infection. Infected fish were screened for dissemination and mortality and imaged on an Olympus confocal microscope at 30-68 hpi, as indicated. **(A and B)** Low temperature infections with TT21 and NRG1^{OEX}. Pooled from 4 independent experiments. **(A)** Dissemination frequencies of TT21 and NRG1^{OEX} infections at 21°C. n=number of live fish screened at time point. **(B)** Kaplan-Meier survival curves display fish survival from TT21-dTomato, NRG1^{OEX}-dTomato and Uninfected control groups at 21°C. Pooled numbers of individual fish are the following: n= 63, 100, and 140 for Uninfected, NRG1^{OEX}, and TT21, respectively. Statistical analysis: (A) Fisher's exact test; (B) Mantel-Cox Log Rank test with Bonferroni correction. *** $p \leq 0.001$, n.s. not significant.

We then sought to ensure that these temperature manipulations by themselves did not affect the activities of shape-locked *C. albicans* strains or overall zebrafish immune function. We used morphology-locked strains to test whether temperature affects the ability of yeast to disseminate or filaments to invade. After infecting fish with the filament-locked UME6^{OEX}-dTomato strain or the yeast-locked NRG1^{OEX}-iRFP strain, we incubated fish at either the yeast-inducing temperature 21°C or the filament-inducing temperature 33°C. As we expected, we found the yeast-dominant NRG1^{OEX} strain continued to disseminate at both low and high temperatures (Figure 2.7A, C). Further, the UME6^{OEX} strain did not disseminate at either temperature, and most fish died by the last time point during infection (Figure 2.7A-D). These results indicate that the functions of yeast and filaments are conserved at temperatures higher and lower than 28°C in zebrafish.

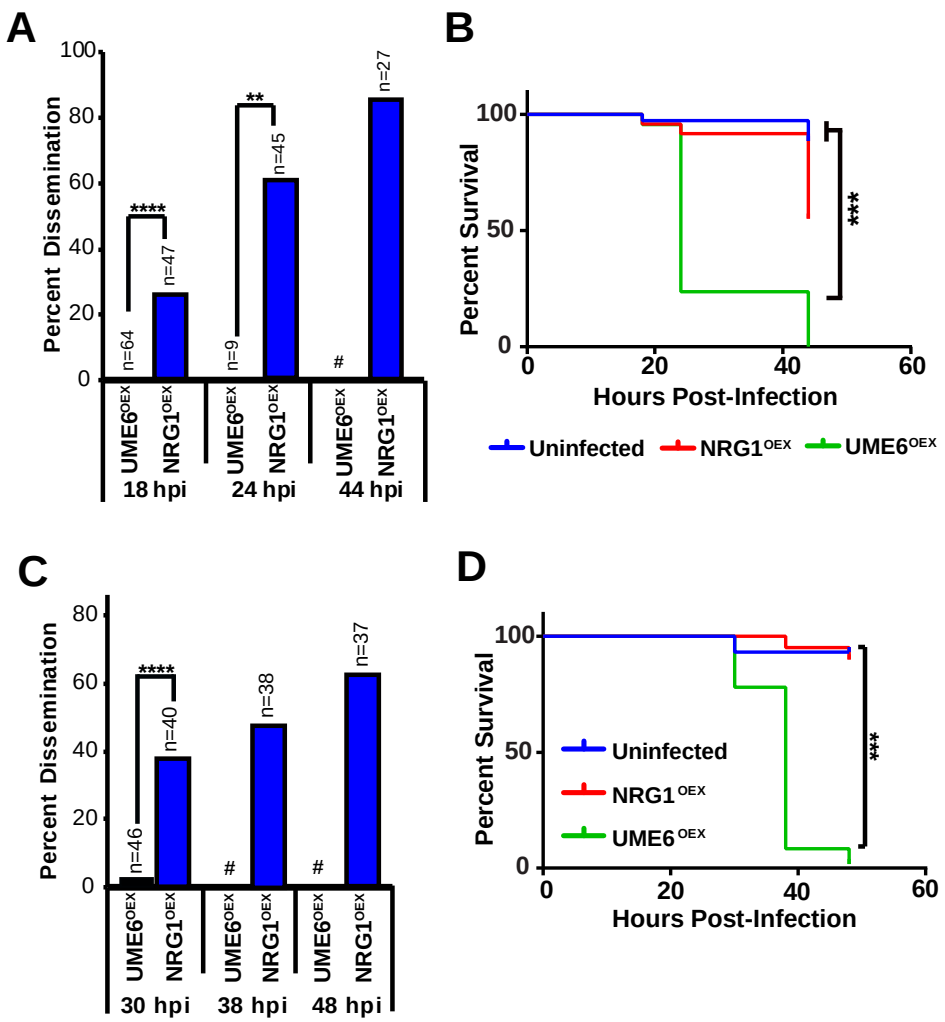

Figure 2.7 Morphology-specific infection phenotypes are conserved at high and low temperatures. Larval AB zebrafish were infected at ~35 hpf with fungi, screened and incubated at either 33°C or 21°C post-infection. Infected fish were screened for dissemination and mortality and imaged on an Olympus confocal microscope at 18, 24, and 44 hpi for 33°C and 30, 38, and 48 hpi for 21°C. **(A and B)** High temperature experiments with UME6^{OEX} and NRG1^{OEX}. Pooled from 4 independent experiments. **(A)** Dissemination frequencies of single infections of UME6^{OEX}-dTomato and NRG1^{OEX}-iRFP at 33°C. n=number of live fish screened at time point. **(B)** Kaplan-Meier curves display fish survival during single infections of UME6^{OEX} and NRG1^{OEX} at 33°C. Pooled numbers of individual fish are the following: n= 78, 49, and 67 for Uninfected, NRG1^{OEX}, and UME6^{OEX}, respectively. **(C and D)** Low temperature infections with UME6^{OEX} and NRG1^{OEX}. Pooled from 4 independent experiments. **(C)** Dissemination frequencies of single infections of UME6^{OEX} and NRG1^{OEX} at 21°C. n=number of live fish screened at time point. **(D)** Kaplan-Meier survival curves display fish survival during single infections of UME6^{OEX} and NRG1^{OEX} at 21°C. Pooled numbers of individual fish are the following: n= 74, 40, and 59 for Uninfected, NRG1^{OEX}, and UME6^{OEX}, respectively. (A) Fisher's exact test; (B) Mantel-Cox Log-Rank test with Bonferroni correction; (C) Fisher's exact test; (D) Mantel-Cox Log Rank test with Bonferroni correction; ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, # = data not analyzed due to more than 90% mortality.

To determine if down-shifting the infection temperature would affect immune homeostasis or a sterile inflammatory response in zebrafish larvae, we assayed the immune system in two different ways. Zebrafish were raised at 33°C until the time of infection (as was done throughout all the experiments described above) and then kept at either 33°C or shifted to 28°C or 21°C for 24 hours to mimic the conditions of infection. At 24 hours post-shift (equivalent developmental stage as 24 hpi), immune homeostasis was assayed by counting overall neutrophil numbers (Figure 2.8) and immune response was quantified by measuring neutrophilic recruitment to a sterile tail wound (Figure 2.9). No differences were found at any temperature measured, suggesting that there are no significant effects of temperature downshift during the time scale of these experiments.

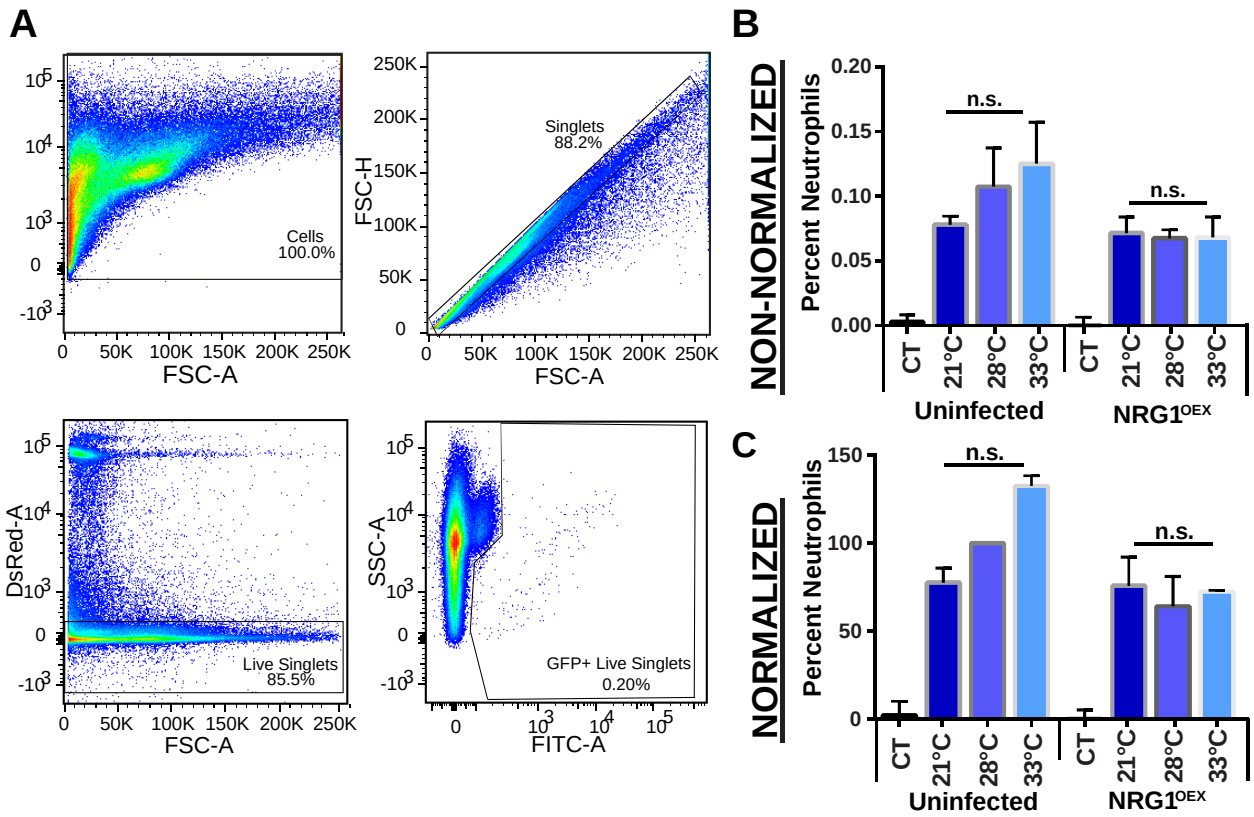

Figure 2.8: Neutrophil ratios do not differ significantly between mock and yeast-infected fish at any temperature. Larval Tg(BAC*mpo*:*GFP*) zebrafish were infected with NRG1^{OEX}-dTomato or PBS and incubated at 21°C, 28°C, or 33°C for 24 hours. Fish from each group were euthanized and homogenized on a cell strainer in PBS/BSA. Fish homogenates were spun down, resuspended in PBS/BSA/Sytox Orange to gate for Live Singlets. Homogenates were then analyzed on an LSRII flow cytometer. **(A, B, and C)** Pooled from 4 independent experiments. n=15-25 fish homogenized per group. **(A)** Representative gates and dot plots of 28°C mock-infected zebrafish at 24 hpi. **(B)** Neutrophil ratios of non-normalized raw neutrophil counts after flow cytometry. **(C)** Neutrophil ratios from (B) were normalized to 28°C mock (PBS) infected fish in each experiment to account for variation in each batch of fish. Statistical analysis: (A) and (B) Kruskal-Wallis test with Bonferroni correction, median with interquartile range displayed. n.s. not significant.

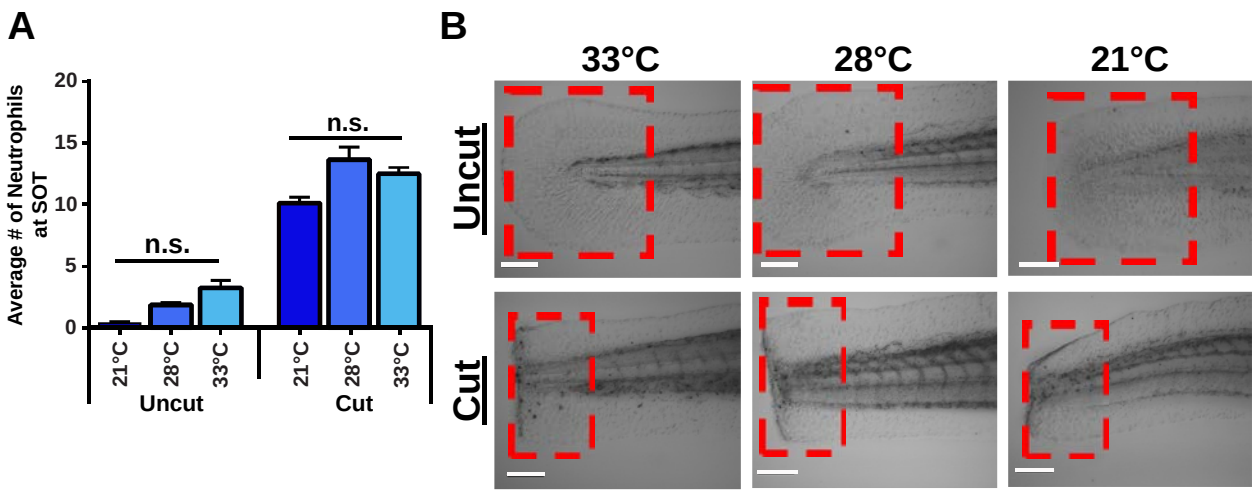

Figure 2.9: Neutrophil numbers quantified at site of transection are similar at all incubation temperatures. At ~35 hpf, fish were anesthetized, injected with PBS and incubated at 21°C, 28°C, and 33°C for 24 hours. At 24 hpi, CUT fish were anesthetized and tail fins were transected with a sterile razor blade. Both CUT and UNCUT groups were incubated for 1 hour at their respective temperatures, euthanized, and fixed in paraformaldehyde overnight at 4°C. Fixed fish were stained with Sudan Black B, washed, and stored in 80% glycerol at 4°C. Neutrophils were quantified at the site of transection using a compound microscope. **(A and B)** Pooled from 2 independent experiments. n=15-20 fish per group. **(A)** Graph shows average number of neutrophils quantified at the site of transection between cut and uncut fish incubated at different temperatures. **(B)** Representative images of Sudan Black stained UNCUT and CUT tail fins from each temperature group. Red dotted rectangles indicate quantified neutrophil regions. Scale bars: 100 μm. Statistical analysis: (A) Kruskal-Wallis test with Bonferroni correction, median with interquartile range displayed. n.s. not significant.

2.3.3 Drug-Regulated Overexpression Strains Change Morphology with Doxycycline in Zebrafish.

Temporally controlling morphology to understand the roles of filaments and yeast has been assessed by using drug-regulatable fungal strains in murine models, including the NRG1^{OEX}, UME6^{OEX}, and TT21 isogenic control strains used in our study (Saville SP *et al*., 2003, Peters BM *et al*., 2014). Although these strains have been used in a murine vaginitis model, their drug-regulatable mechanisms have yet to be tested in zebrafish in the context of disseminated, invasive infections. To test the feasibility of altering morphology in these infections with a drug, we infected fish with TT21 or NRG1^{OEX}, with or without the presence of doxycycline, and

analyzed dissemination frequencies, mortality, and relative yeast presence at 21°C, 28°C, and 33°C. The NRG1^{OEX} strain is engineered to overexpress the *NRG1* gene under control of a drug-regulatable promoter, and can be turned off to express a “wildtype” phenotype with tetracycline or its derivative, doxycycline (Figure 2.10A-B). In contrast, the TT21 isogenic control has an empty vector in place of a gene, and maintains a similar phenotype regardless of drug presence (Figure 2.10C) (Peters BM *et al*., 2014). As we expected, the TT21/TT21+DOX wildtype infections resulted in similar low dissemination, but high mortality and filament presence at 28°C and 33°C (Figure 2.11A-C, 2.12A-C). Further, during NRG1^{OEX} infection with doxycycline (NRG1^{OEX}+DOX) at 28°C and 33°C, we similarly found a filament-dominant infection resulting in high mortality and low dissemination, with the exception of the 24-hour time point for dissemination at 33°C (Figure 2.11A-C, 2.12A-C). These results suggest that the NRG1^{OEX} overexpression can be turned off in zebrafish using doxycycline, resulting in a similar phenotype as the TT21/TT21+DOX wildtype infections at moderate and high temperatures. When we analyzed the TT21+DOX and NRG1^{OEX}+DOX infections at the yeast-inducing 21°C, we found that both strains were able to produce yeast, leading to a steady increase in dissemination over time with prolonged survival, similar to the wildtype TT21 strain (Figure 2.13A-C). Thus, doxycycline does not significantly alter the ability of the fungus to regulate its morphology in the presence of at least one environmental cue. Overall, we find these drug-regulatable strains, specifically TT21+DOX and NRG1^{OEX}+DOX, display similar dissemination and mortality phenotypes as the unmanipulated TT21 isogenic control at all temperatures tested in the zebrafish. Further, the yeast-locked NRG1^{OEX} strain continues to disseminate without high rates of death at all temperatures, as expected from previous experiments. Thus, these strains provide additional evidence of the roles of both yeast and filaments during invasive, disseminated infections, while validating their applicability in the zebrafish.

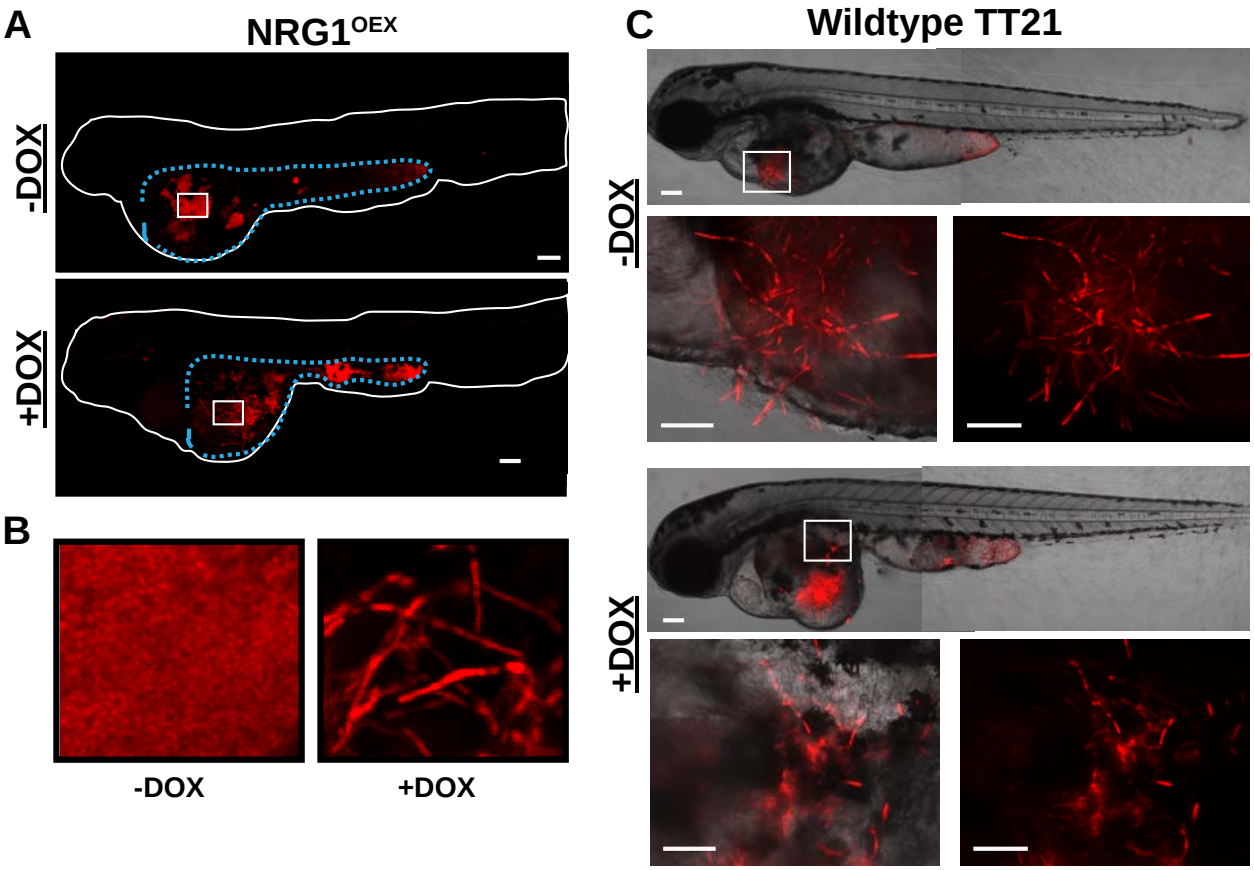

Figure 2.10: Regulating overexpression strains TT21 and NRG1^{OEX} with doxycycline is feasible in zebrafish. Larval AB zebrafish were infected at ~35 hpf with fungi, screened on a Zeiss epifluorescence microscope for inoculum, and incubated at 28°C for experimental duration. Infected fish were screened and imaged on an Olympus confocal microscope at 30 hpi (NRG1^{OEX}/NRG1^{OEX}+DOX) or 24 hpi (TT21/TT21+DOX). **(A)** Representative images of NRG1^{OEX}-dTomato ± doxycycline (DOX) during infection in the zebrafish yolk. Outline of whole fish in white, blue dotted line outlines yolk. **(B)** Insets of (A) yolk infections of NRG1^{OEX} and NRG1^{OEX}+DOX. **(C)** Representative images of TT21-dTomato ± DOX infections, with yolk insets. Scale bars: whole fish = 100 µm, insets = 50 µm.

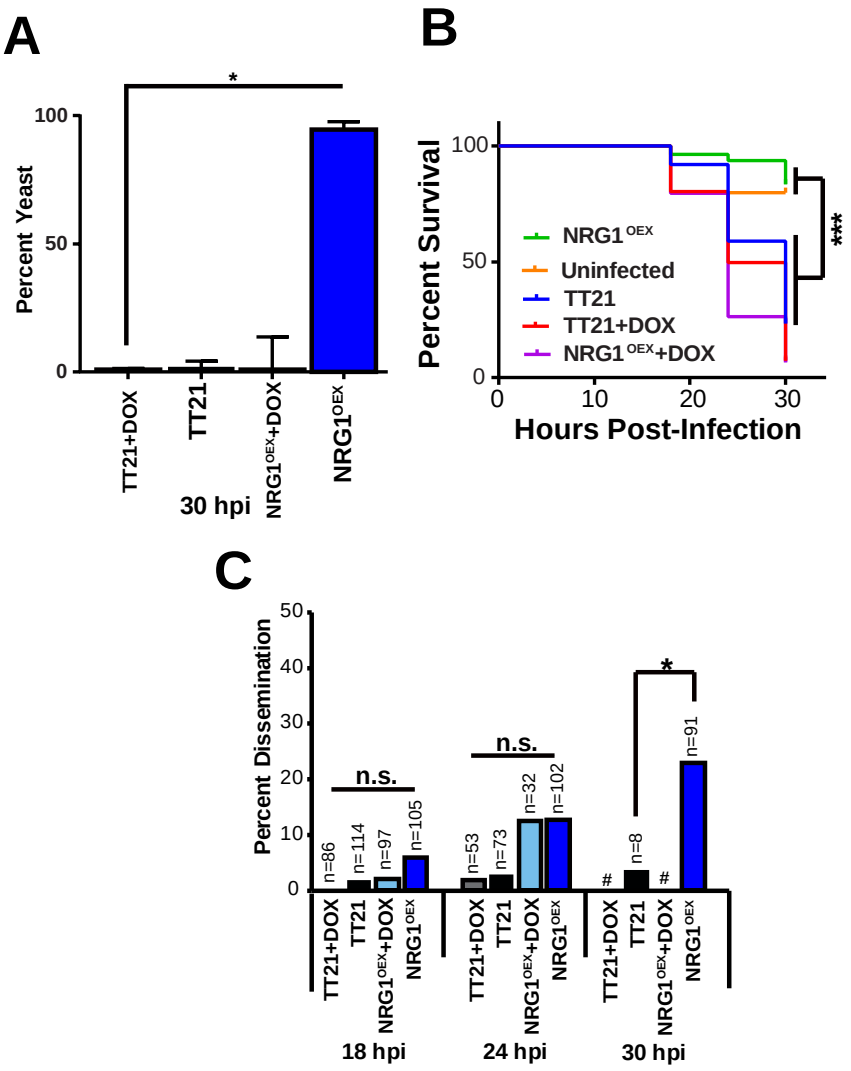

Figure 2.11: Manipulating overexpression strains with doxycycline results in similar morphology, dissemination, and mortality phenotypes as TT21 infections at 33°C. Larval AB zebrafish were infected at ~35 hpf with fungi, screened, and incubated at 33°C. Infected fish were screened for dissemination and mortality and imaged on an Olympus confocal microscope at 18, 24 and 30 hpi. At 30 hpi, fish were euthanized for morphology quantification as described in Materials and Methods. **(A, B, and C)** Single infections of TT21 and NRG1^{OEX} ± doxycycline at 33°C. Pooled from 4 independent experiments. **(A)** Percent yeast during TT21, TT21^{DOX}, NRG1^{OEX}, and NRG1^{OEX}+DOX single infections at 33°C. **(B)** Kaplan-Meier curve displays fish survival during single infections of TT21, TT21^{DOX}, NRG1^{OEX}, and NRG1^{OEX}+DOX at 33°C. Pooled numbers of individual fish are the following: n= 59, 124, 107, 109, and 122 for Uninfected, TT21, TT21+DOX, NRG1^{OEX}, and NRG1^{OEX}+DOX infections, respectively. **(C)** Dissemination frequencies of single infections of TT21, TT21+DOX, NRG1^{OEX}, and NRG1^{OEX}+DOX at 33°C. n=number of live fish screened at time point. Statistical analysis: (A) Kruskal-Wallis test with Bonferroni correction, median with interquartile range displayed; (B) Mantel-Cox Log Rank test with Bonferroni correction; (C) Fisher's exact test with Bonferroni correction. * p≤0.05, *** p≤0.001, n.s. not significant, #=data not analyzed due to more than 90% mortality.

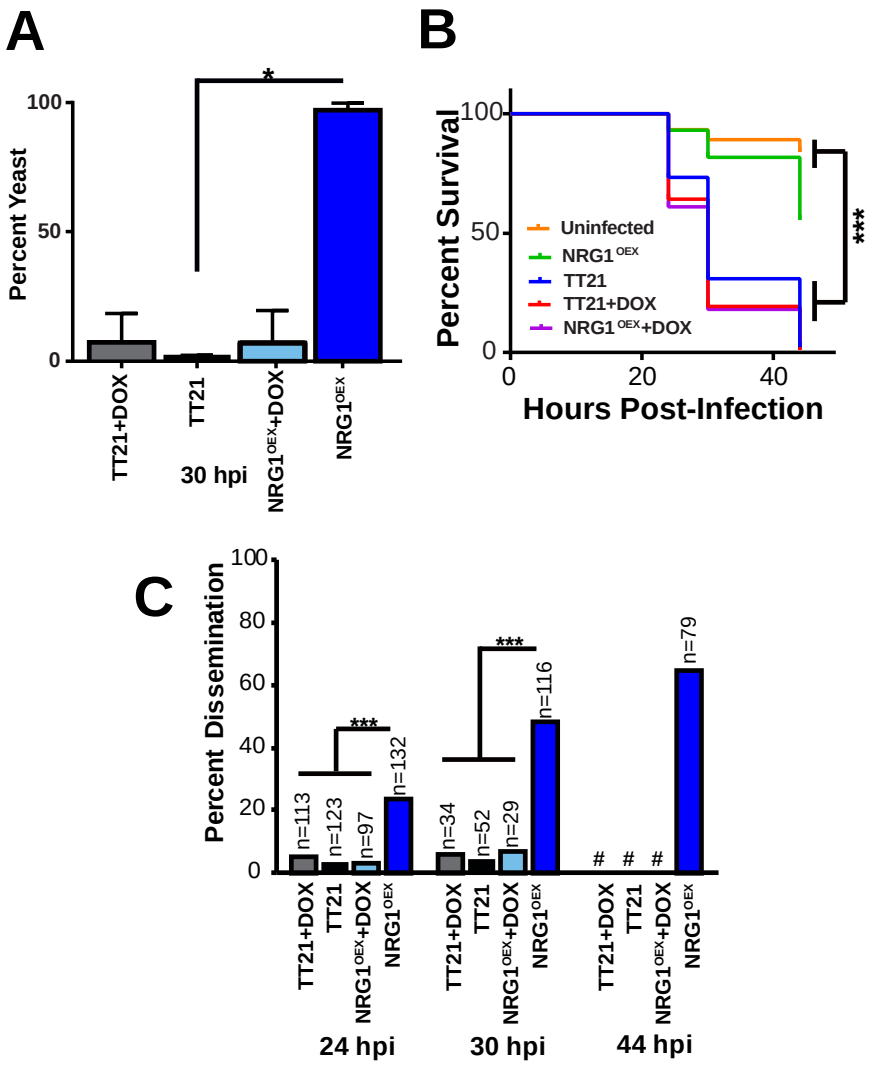

Figure 2.12 Manipulating overexpression strains with doxycycline results in similar morphology, dissemination, and mortality phenotypes as TT21 infections at 28°C. Larval AB zebrafish were infected at ~35 hpf with fungi, screened, and incubated at 28°C. Infected fish were screened for dissemination and mortality and imaged on an Olympus confocal microscope at 24, 30 and 44 hpi. At 30 hpi, fish were euthanized for morphology quantification as described in Materials and Methods. **(A, B, and C)** Single infections of TT21 and NRG1^{OEX} ± doxycycline at 28°C. Pooled from 5 independent experiments. **(A)** Percent yeast during TT21, TT21+DOX, NRG1^{OEX}, and NRG1^{OEX}+DOX single infections at 28°C. **(B)** Kaplan-Meier curve displays fish survival during single infections of TT21, TT21+DOX, NRG1^{OEX}, and NRG1^{OEX}+DOX at 28°C. Pooled numbers of individual fish are the following: n= 74, 168, 176, 142, and 159 for Uninfected, TT21, TT21+DOX, NRG1^{OEX}, and NRG1^{OEX}+DOX infections, respectively. **(C)** Dissemination frequencies of single infections of TT21, TT21+DOX, NRG1^{OEX}, and NRG1^{OEX}+DOX at 28°C. n=number of live fish screened at time point. Statistical analysis: (A) Kruskal-Wallis test with Bonferroni correction, median with interquartile range displayed; (B) Mantel-Cox Log Rank test with Bonferroni correction; (C) Fisher's exact test with Bonferroni correction. * p≤0.05, *** p≤0.001, #=data not analyzed due to more than 90% mortality.

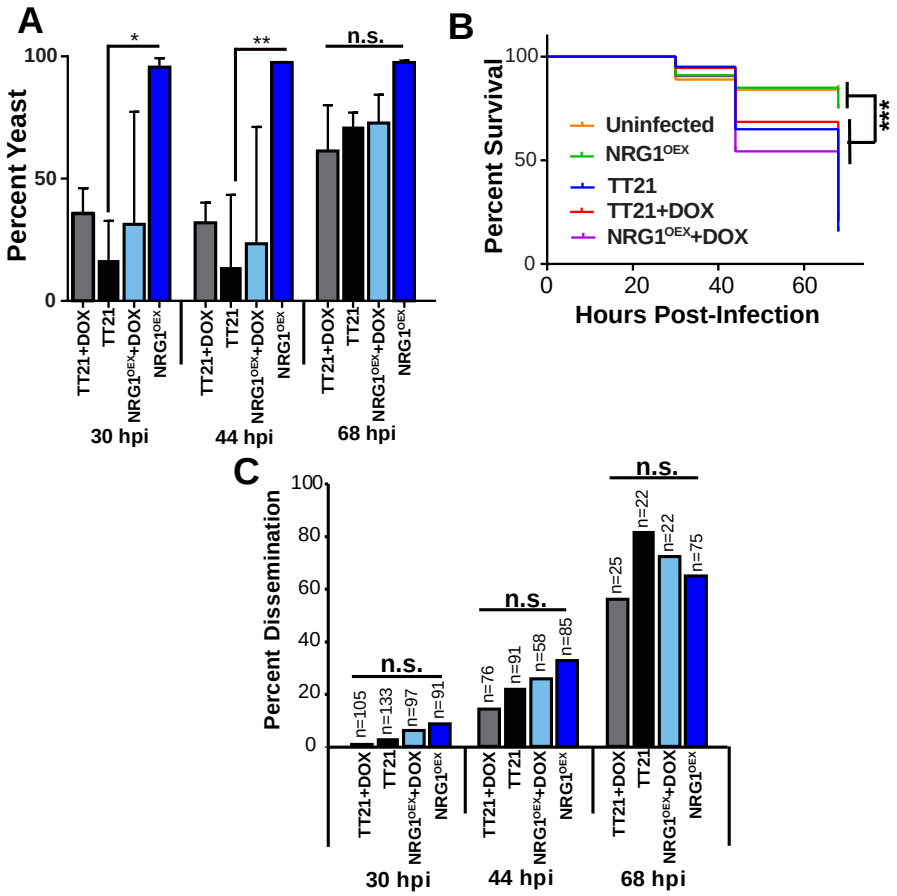

Figure 2.13 Manipulating overexpression strains with doxycycline results in similar morphology, dissemination, and mortality phenotypes as TT21 infections at 21°C. Larval AB zebrafish were infected at ~35 hpf with fungi, screened, and incubated at 21°C. Infected fish were screened for dissemination and mortality and imaged on an Olympus confocal microscope at 30, 44 and 68 hpi. At all time points, randomly chosen fish were euthanized for morphology quantification as described in Materials and Methods. **(A, B, and C)** Single infections of TT21 and NRG1^{OEX} ± doxycycline at 21°C. Pooled from 4 independent experiments. **(A)** Percent yeast during TT21, TT21+DOX, NRG1^{OEX}, and NRG1^{OEX}+DOX single infections at 21°C. **(B)** Kaplan-Meier curve displays fish survival during single infections of TT21, TT21+DOX, NRG1^{OEX}, and NRG1^{OEX}+DOX at 21°C. Pooled numbers of individual fish are the following: n=63, 140, 111, 100, and 107 for Uninfected, TT21, TT21+DOX, NRG1^{OEX}, and NRG1^{OEX}+DOX infections, respectively. **(C)** Dissemination frequencies of single infections of TT21, TT21+DOX, NRG1^{OEX}, and NRG1^{OEX}+DOX at 21°C. n=number of live fish screened at time point. Statistical analysis: (A) Kruskal-Wallis test with Bonferroni correction, median with interquartile range displayed; (B) Mantel-Cox Log Rank test with Bonferroni correction; (C) Fisher's exact test with Bonferroni correction. * p≤0.05, ** p≤0.01, *** p≤0.001, n.s. not significant.

2.3.4 Increased Filament Production Moderately Correlates with Increased Mortality, While Increased Yeast Production Does Not Always Correlate with High Dissemination.

Studies have suggested that a specific threshold of yeast is needed in the gut for dissemination to occur in a murine model of gastrointestinal infection (Kennedy MJ and Volz PA, 1985, Kennedy MJ and Volz PA, 1983). In addition, the dissemination frequency of the wildtype TT21 at 21°C suggests that a certain yeast:filament ratio may be necessary for dissemination to occur, especially at later time points. The continued growth of filaments during 28°C wildtype infections also seems to correlate with an increase in mortality over time. However, to our knowledge, no studies have analyzed the importance of morphotype ratio in dissemination or mortality *in vivo*. Thus, we sought to investigate whether the morphotype-function relationships we observe are positively correlated to the ratio of filaments:yeast or yeast:filaments present during infection. Infecting with TT21 or NRG1^{OEX} at all temperatures, with or without the presence of doxycycline, we analyzed different yeast:filament ratios compared to mortality or dissemination at 30 hpi, the only similar time point for all temperatures tested. Interestingly, we found that an increase in filament:yeast ratio moderately correlates with an increase in mortality (Figure 2.14A). However, the presence of 21°C wildtype data (an early time point in those experiments; green triangle/circle/diamond) results in a relatively low mortality rate, although the infection is filament dominant at that time point. This is due to the slowed infection progression at this temperature. Overall, we find that an increase in filament growth from ~60-80% (values for wildtypes at 21°C) to 100% results in an increase in mortality. In the context of yeast and dissemination, we found a slightly weaker correlation between a high yeast:filament ratio and dissemination frequency, suggesting that a higher presence of yeast to filaments does not automatically lead to a higher dissemination frequency (Figure 2.14B). In fact, we find that dissemination of the yeast-dominant NRG1^{OEX} strain differs at 30 hpi among temperatures, with 21°C having the lowest frequency of dissemination, and this is again due to the slowed progression of infection at this temperature (Figure 2.14B). Thus, a higher yeast presence does

not directly correlate to a higher dissemination frequency in these infections. Overall, we find that an increase in filament ratio leads to a moderately increased mortality in fish, while an increase in yeast production does not always lead to a highly disseminated infection.

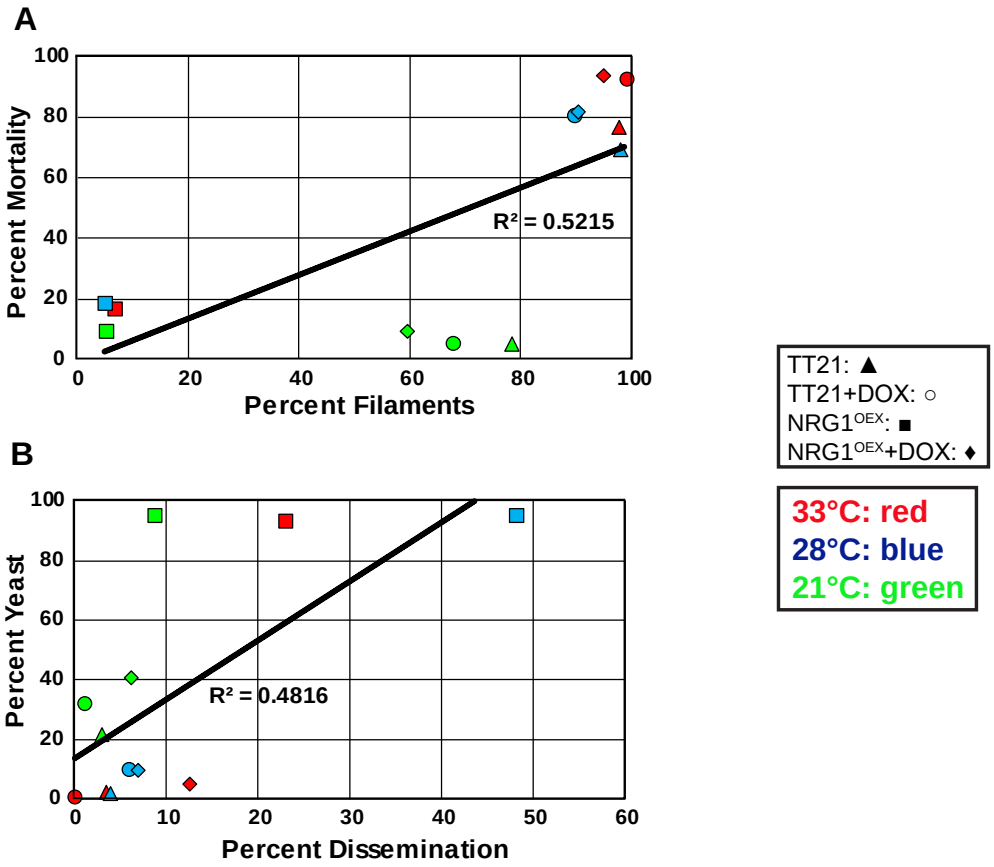

Figure 2.14: Increased filament production moderately correlates with increased mortality, while increased yeast production does not always correlate with high dissemination. Larval AB zebrafish were infected at ~35 hpf with either TT21 or NRG1^{OEX} ± doxycycline (DOX), screened on a Zeiss epifluorescence microscope for inoculum, and incubated at 33°C, 28°C, or 21°C for experimental duration. At 30 hpi, infected fish from each group were euthanized and flattened onto a glass slide under a glass coverslip to quantify morphology. Morphotype ratios are compared to dissemination frequencies and mortality at 30 hpi for each strain at each infection temperature, and tested for correlation. **(A and B)** 33°C data pooled from 4 independent experiments, 28°C data pooled from 5 independent experiments, 21°C data pooled from 4 independent experiments. Triangles = TT21, circles = TT21+DOX, squares = NRG1^{OEX}, diamonds = NRG1^{OEX}+DOX. Infections at 33°C labeled red, infections at 28°C labeled blue, infections at 21°C labeled green. **(A)** Scatter plot represents filament:yeast ratio versus mortality rate of TT21 and NRG1^{OEX} ± DOX infections at all temperatures. Best fit line and R^2 value included. **(B)** Scatter plot showing yeast:filament ratio versus dissemination frequencies for TT21 and NRG1^{OEX} ± DOX infections at all temperatures. Best fit line and R^2 value included.

2.3.5 Morphotypes Do Not Synergize to Enhance Infection Outcome During Mixed Infections.

The quantitative results from our previous experiments using shape-locked strains suggested the possibility that invasion and dissemination are driven by the presence of a specific fungal shape, rather than the process of dimorphic transition alone. While it has been long hypothesized that the presence of both shapes or a dimorphic transition leads to enhanced pathogenesis, no studies have tested whether the simultaneous presence of each shape can substitute for the ability to switch form. To test if a mix of shapes is sufficient to make up for the inability to change shape, we infected fish with a mix of filament-locked ($UME6^{OEX}$) and yeast-locked ($NRG1^{OEX}$) strains and compared dissemination and lethality between these mixed and single strain infections (Figure 2.15A). These experiments were enabled by our previous finding that a two-fold change in the infectious dose of either strain did not quantitatively affect the efficiency of yeast dissemination or filament invasion during single infections (Figures 2.4, 2.5). Surprisingly, in these mixed infections we found similar dissemination frequencies between mixed ($UME6^{OEX}$+$NRG1^{OEX}$) and single ($NRG1^{OEX}$) infections (Figure 2.15B). Consistent with these findings, we also measured similar dissemination frequencies in infections with SC5314 and hypofilamentous mutants (Figure 2.16A-B). Further, we found similar dissemination frequencies in $NRG1^{OEX}$/$UME6^{OEX}$/mixed infections at both 33°C and 21°C, although infection progression was slowed at the low temperature, potentially due to the slowed fungal growth that occurs at this temperature (Figure 2.17A, C, 2.18). These results suggest that filaments do not affect the ability of yeast to disseminate. Interestingly, we found less mortality in mixed infections compared to filament-dominant infections, suggesting that yeast do somehow affect the mortality associated with filament invasion (Figure 2.15C, 2.16C, 2.17B, D). Overall, these results suggest that morphotypes do not synergize to enhance each other's activities.

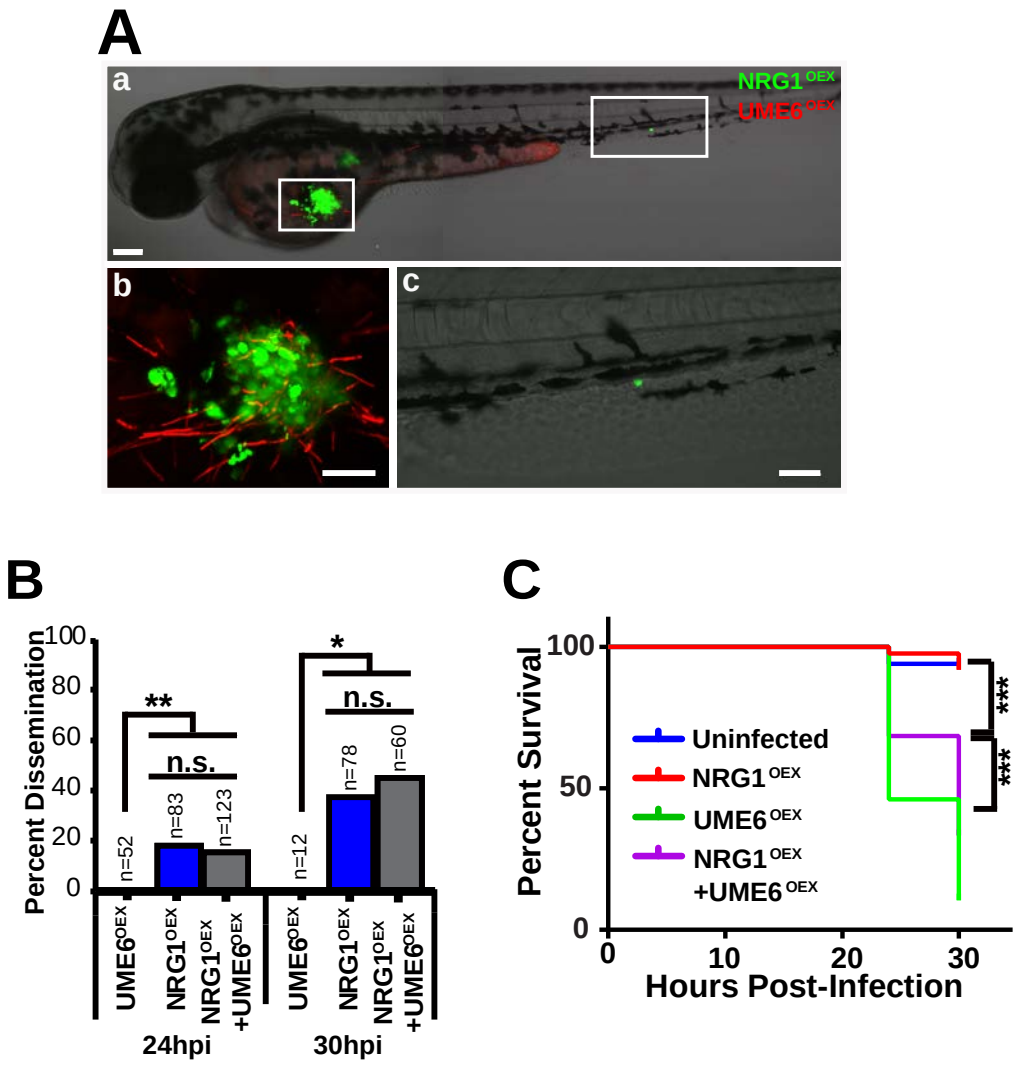

Figure 2.15: Morphotypes do not affect each other's abilities to disseminate or invade in mixed overexpression strain infections. Larval AB zebrafish were infected at ~35 hpf with fungi, screened for inoculum, and incubated at 28°C. Infected fish were screened for dissemination and mortality and imaged on an Olympus confocal microscope at 24 or 30 hpi. **(A)** Representative image (a) and insets (b and c) of NRG1^{OEX}-NEON and UME6^{OEX}-dTomato mixed infection at 24 hpi. (b) inset of yeast and filaments in yolk. (c) inset of disseminated yeast in tail. **(B and C)** Single and mixed infections with NRG1^{OEX} and UME6^{OEX}. Pooled from 4 independent experiments. **(B)** Dissemination frequencies between single and mixed infections of NRG1^{OEX} and UME6^{OEX} strains. n=number of live fish screened at time point. **(C)** Kaplan-Meier survival curves display fish survival during NRG1^{OEX}, UME6^{OEX}, and mixed morphology infections. Pooled numbers of individual fish are the following: n= 65, 85, 113, and 180 for Uninfected, NRG1^{OEX}, UME6^{OEX}, and mixed infections, respectively. Scale bars: whole fish = 100 µm, insets = 50 µm. Statistical analysis: (B) Fisher's exact test with Bonferroni correction; (C) Mantel-Cox Log Rank test with Bonferroni correction. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, n.s. not significant.

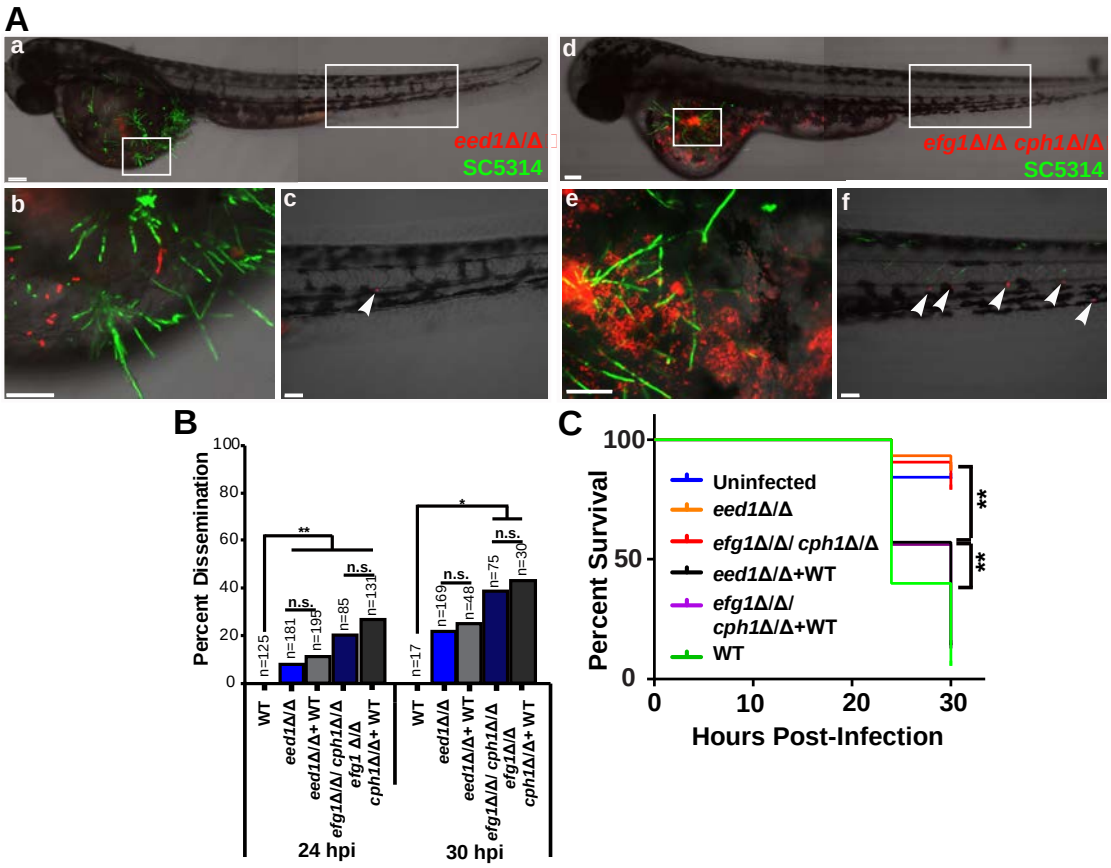

Figure 2.16: Morphotypes do not synergize to enhance dissemination or invasion in mixed hypofilamentous mutant and wildtype infections. Larval AB zebrafish were infected at ~35 hpf with fungi, screened on a Zeiss epifluorescence microscope for inoculum, and incubated at 28°C for experimental duration. Infected fish were screened and imaged on an Olympus confocal microscope at 24 or 30 hpi for dissemination and mortality. **(A)** Representative images of SC5314-GFP and mutant (*eed1*Δ/Δ-dTomato or *efg1*Δ/Δ/*cph1*Δ/Δ-dTomato) coinfections at 24 hpi. (a, d) Representative images of SC5314-GFP+*eed1*Δ/Δ-dTomato and SC5314-GFP+*efg1*Δ/Δ/*cph1*Δ/Δ-dTomato infections. (b, e) insets of each mixed infection in the yolk. (c, f), insets of yeast dissemination in tail of each mixed infection. White arrowheads indicate disseminated yeast. **(B and C)** Single and mixed infections with SC5314, *eed1*Δ/Δ, and *efg1*Δ/Δ/*cph1*Δ/Δ. Pooled from 9 independent experiments. **(B)** Dissemination frequencies between single and mixed infections of hypofilamentous mutants (*eed1*Δ/Δ-dTomato, *efg1*Δ/Δ/*cph1*Δ/Δ-dTomato) and filament-dominant wildtype (SC5314-GFP) strains. n=number of live fish screened at time point. **(C)** Kaplan-Meier survival curves display fish survival during SC5314, *eed1*Δ/Δ, *efg1*Δ/Δ/*cph1*Δ/Δ, and mixed morphology infections. Pooled numbers of individual fish are the following: n= 68, 314, 194, 342, 94, and 233 for Uninfected, SC5314, *eed1*Δ/Δ, SC5314+*eed1*Δ/Δ, *efg1*Δ/Δ/*cph1*Δ/Δ, and SC5314+*efg1*Δ/Δ/*cph1*Δ/Δ, respectively. Scale bars: whole fish = 100 μm, insets = 50 μm. Statistical analysis: (B) Fisher's exact test with Bonferroni correction; (C) Mantel-Cox Log Rank test with Bonferroni correction. * p≤0.05, ** p≤0.01, n.s. not significant.

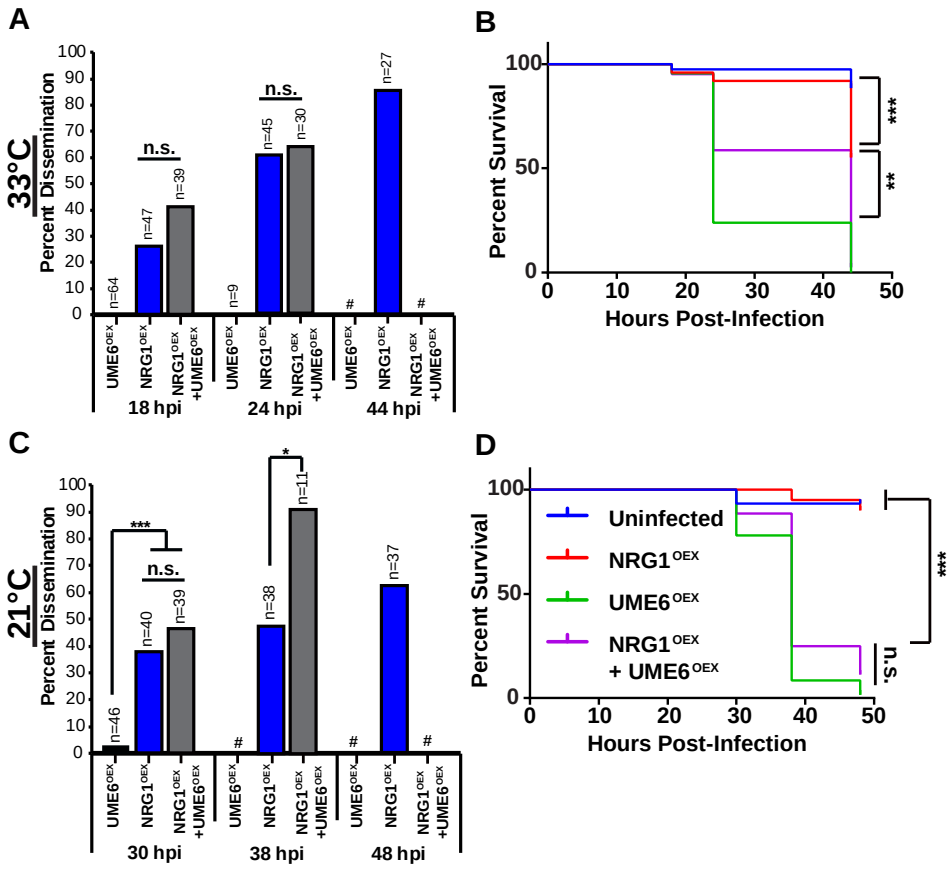

Figure 2.17: Single and mixed morphology infections of overexpression strains produce similar levels of dissemination and mortality at 33°C and 21°C. Larval AB zebrafish were infected at ~35 hpf with fungi, screened on a Zeiss epifluorescence microscope for inoculum, and incubated at 33°C or 21°C for experimental duration. Infected fish were screened and imaged on an Olympus confocal microscope for dissemination and mortality. **(A and B)** Single and mixed infections with NRG1^{OEX} and UME6^{OEX} at 33°C. Pooled from 4 independent experiments. **(A)** Dissemination frequencies of single and mixed infections of UME6^{OEX}-dTomato and NRG1^{OEX}-iRFP at 33°C. n=number of live fish screened at time point. **(B)** Kaplan-Meier curves display fish survival during single and mixed infections of UME6^{OEX} and NRG1^{OEX} at 33°C. Pooled numbers of individual fish are the following: n= 78, 67, 49, and 41 for Uninfected, UME6^{OEX}, NRG1^{OEX}, and mixed infections, respectively. **(C and D)** Single and mixed infections with NRG1^{OEX} and UME6^{OEX} at 21°C. Pooled from 4 independent experiments. **(C)** Dissemination frequencies of single and mixed infections of UME6^{OEX} and NRG1^{OEX} at 21°C. n=number of live fish screened at time point. **(D)** Kaplan-Meier curves display fish survival during single and mixed infections of UME6^{OEX} and NRG1^{OEX} at 21°C. Pooled numbers of individual fish are the following: n= 74, 59, 40, and 44 for Uninfected, UME6^{OEX}, NRG1^{OEX}, and mixed infections, respectively. Statistical analysis: (A) Fisher's exact test with Bonferroni correction; (B) Mantel-Cox Log Rank test with Bonferroni correction; (C) Fisher's exact test with Bonferroni correction; (D) Mantel-Cox Log Rank test with Bonferroni correction. * p≤0.05, ** p≤0.01, *** p≤0.001, n.s. not significant, #=data not analyzed due to more than 90% mortality.

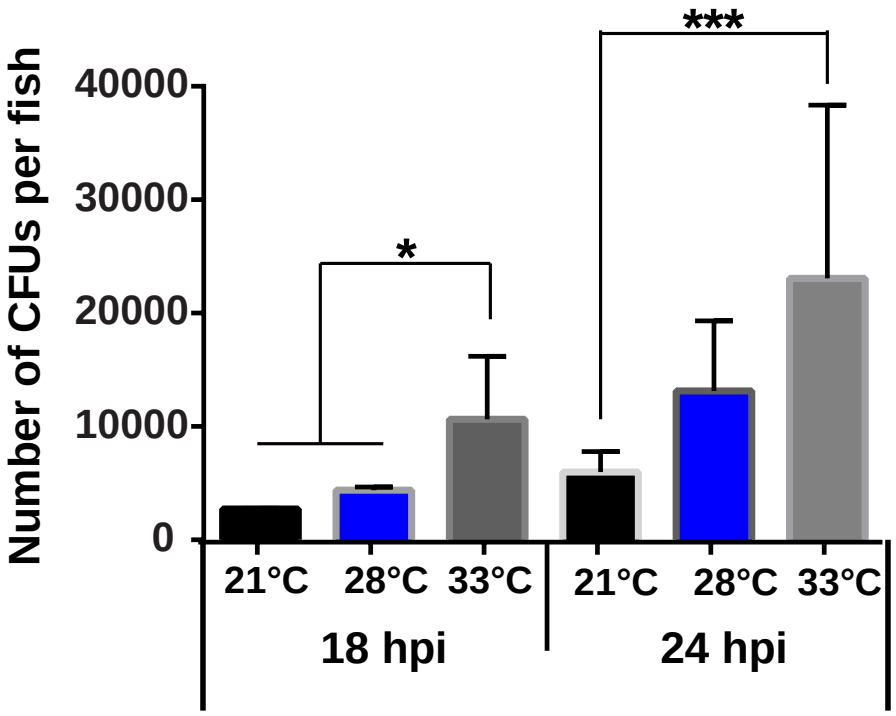

Figure 2.18: *C. albicans* grows at distinct speeds during altered temperature incubation. Larval AB fish were infected at ~35 hpf with NRG1^{OEX}-dTomato and incubated at 21°C, 28°C, or 33°C for 18-24 hours. At each time point, 5 fish per group were euthanized and homogenized in PBS before diluting and plating onto YPD media supplemented with antibiotics. Plates were grown overnight, visible colonies quantified, and CFUs were calculated per fish. Pooled from 3 independent experiments per time point. NRG1^{OEX} colony-forming units (CFUs) quantified at 18 and 24 hpi incubated at 21°C, 28°C, and 33°C. Statistical analysis done using Kruskal-Wallis test with Bonferroni correction. * $p \leq 0.05$, *** $p \leq 0.001$.

Our results suggesting that yeast and filaments do not enhance each other during infection was unexpected and drove us to further investigate this lack of synergy. We postulated that the relative number of filament- or yeast-locked cells in the mixed infectious dose may affect dissemination or invasion/mortality. To test this idea, we split mixed infections between low (1-10 cells) or high (11-20 cells) filament or yeast inocula and analyzed the impact of these dosages. As noted previously, in single infections we found no significant differences in these two different infection doses (Figures 2.4, 2.5). In the context of yeast function, we found that filament inoculum size had no impact on the ability of yeast to disseminate or proliferate in the yolk (Figure 2.19A-B). Filament invasion also did not impact the ability to disseminate (Figure

2.19C). In the context of filament function, we found that yeast inoculum size does not affect survival or impact the ability of filaments to invade tissue (Figure 2.20A-B). These results are consistent with the idea that dissemination and invasion are insensitive to the presence of both morphotypes. While comparison of mixed and mono-infections (Figures 2.15, 2.16, 2.17) suggests that the presence of yeast limits filament-driven mortality, these experiments suggest that a more subtle alteration in yeast dose (between 1-10 and 11-20 cells) does not significantly impact survival.

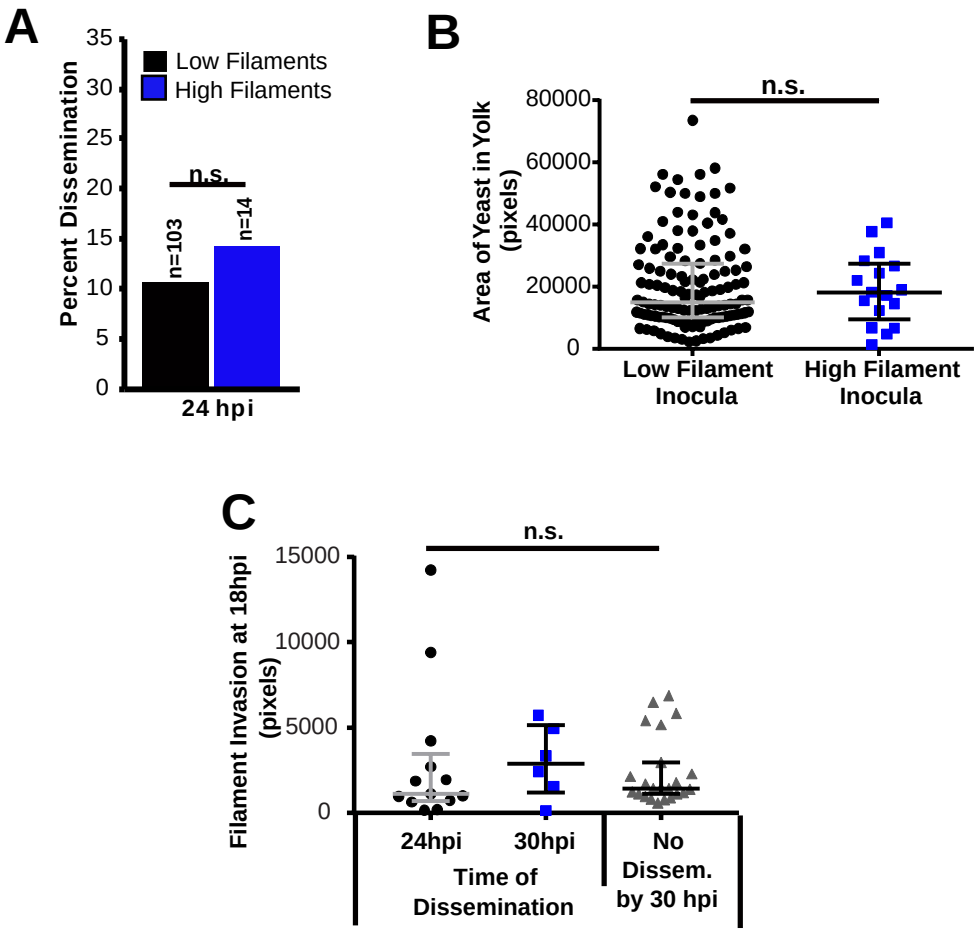

Figure 2.19: Filaments and invasion do not significantly alter the ability of yeast to disseminate or grow in the yolk. Larval AB zebrafish were infected at ~35 hpf with mixed infections of UME6^{OEX}-dTomato and NRG1^{OEX}-NEON, screened for exact inoculums, and individually placed in 48-well plates. Fish were kept at 28°C for the duration of the experiment. At 18 hpi, fish yolks were imaged for filament invasion and yeast growth, and imaged fish were followed out to 24 and 30 hpi for mortality and dissemination. **(A, B, and C)** Dissemination and yeast levels in mixed infections with different filament inocula. Pooled from 7 independent experiments. **(A)** Percent dissemination of mixed infections with low (1-10) or high (11-20) filament inocula. n=number of live fish screened at time point. **(B)** Yeast levels, quantified by pixels in ImageJ, in fish with either low (1-10) or high (11-20) filament inocula (black circles are low inocula, blue squares are high inocula). **(C)** Level of invasion at 18 hpi in fish that go on to have disseminated infection at 24 hpi or 30 hpi, compared to those that remain without dissemination by 30 hpi (black circles are dissemination at 24 hpi, blue squares are dissemination at 30 hpi, grey triangles are non-disseminated). Filament invasion was quantified in confocal images taken at 18 hpi and dissemination was scored at 24 and 30 hpi. Statistical analysis: (A) Fisher's exact test; (B) Mann-Whitney U test, median with interquartile range displayed; (C) Kruskal-Wallis test with Bonferroni correction, median with interquartile range displayed. n.s. not significant.

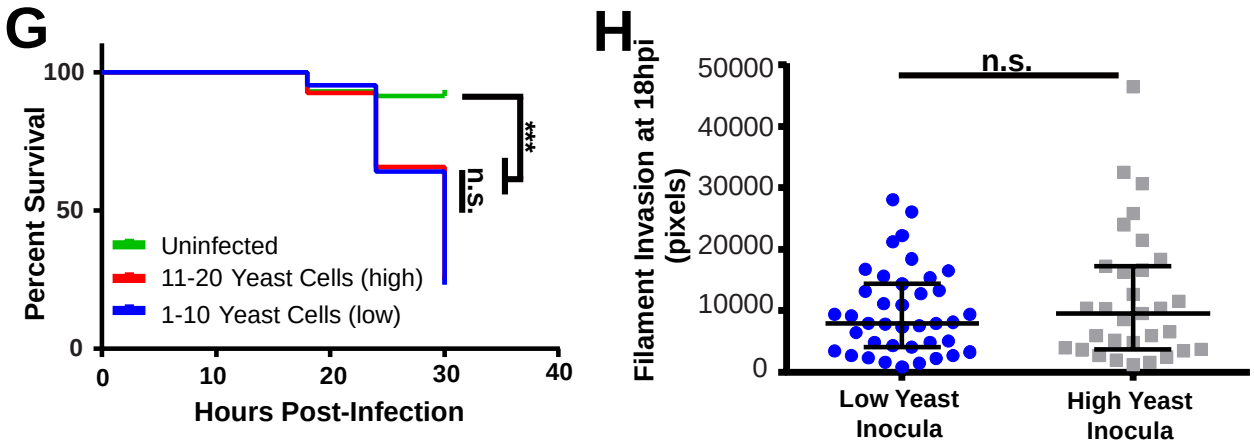

Figure 2.20: Yeast do not affect the ability of filaments to kill fish or to invade during mixed infections. Larval AB zebrafish were infected at ~35 hpf with single infections of UME6^{OEX}-dTomato, screened for exact inoculums, and individually placed in 48-well plates. Fish were kept at 28°C for the duration of the experiment. At 18 hpi, fish yolks were imaged for filament invasion and imaged fish were followed out to 24 and 30 hpi for mortality. **(A and B)** Invasion and mortality in mixed infections with different yeast inocula. Pooled from 4-7 independent experiments. **(A)** Kaplan-Meier survival curves display fish survival during infection with low (1-10) or high (11-20) NRG1^{OEX} inocula. Pooled numbers of individual fish are the following: n= 115, 517, and 257 for Uninfected, mixed+low NRG1^{OEX}, and mixed+high NRG1^{OEX}, respectively. **(B)** Filament invasion at 18 hpi, in pixels as quantified by ImageJ (FIJI), during infections with low or high yeast inocula (blue circles are low yeast inocula, grey squares are high yeast inocula). Statistical analysis: (A) Mantel-Cox Log Rank test with Bonferroni correction; (B) Mann-Whitney U test, median with interquartile range displayed. *** $p \leq 0.001$, n.s. not significant.

CHAPTER 3

DISCUSSION

Invasive, disseminated *C. albicans* infections claim up to 30-50% of those diagnosed (Gudlaugsson O *et al*., 2003, Horn DL *et al*., 2009). *C. albicans* has a pleomorphic ability to switch between yeast and filament growth that is a crucial virulence factor during these deadly infections. It has been long-hypothesized that yeast drive dissemination while filaments are primarily invasive, but technical limitations have prevented the visualization of these processes during infection. We use a simplified, transparent vertebrate infection model to probe the individual and combined abilities of both yeast and filament forms of *C. albicans*. Our findings in the larval zebrafish suggest that yeast and filaments have specialized, independent roles during tissue-to-bloodstream spread in candidiasis. Infections with genetically-modified strains and wildtype infections during temperature shifts all show that yeast disseminate, in contrast to filaments that invade tissue and ultimately kill the host. Mixed infections provide further evidence of each of these roles, and surprisingly demonstrate that the two morphotypes, with defined numbers of morphology-locked strains, do not synergize to enhance infection progression. Overall, our study confirms the long-suspected roles of each morphotype during infection, and demonstrates that there is little influence of one morphotype on the other during disease development.

Zebrafish provide a platform to answer questions that cannot be investigated in murine or *in vitro* models during host-*C. albicans* interactions. Transparency is a unique trait of this vertebrate that allowed us to visualize all fungal cells throughout the host at high resolution. This enabled non-invasive quantification of morphology, invasion, yeast proliferation, and dissemination frequency, of which only invasion has been previously quantified *in vitro* through damage assays (Sanchez AA *et al*., 2004). Longitudinal imaging of individual fish enabled us to link invasion directly with mortality and test connections between inoculum size and pathogenesis, similar to simpler inoculum size-mortality correlations that have been analyzed in

mice (MacCallum DM and Odds FC, 2005). Another advantage of the zebrafish is the cost-effectiveness of husbandry (Lieschke GJ and Currie PD, 2007), which permitted the testing of multiple fungal strains and environmental conditions using the same infection protocol. Furthermore, we could take advantage of the flexibility of zebrafish for growth at different temperatures to manipulate *C. albicans* morphology, which has so far only been analyzed *in vitro* (Shapiro RS *et al*., 2009). In the future, we can take advantage of multiple transgenic lines (Ellett F *et al*., 2011, Renshaw SA *et al*., 2006) to examine the role of immune response in yeast dissemination, prevention of invasion, and the unexpected contributions of yeast to reduced mortality during coinfections.

Balancing these unique advantages, it is important to note a few differences in our model from murine infections. Due to the small size of the fish, *C. albicans* filaments can damage a significant percentage of fish quite rapidly. Thus, only a few filament cells are sufficient to kill a larval fish, compared to a much larger dose necessary in mice (MacCallum DM and Odds FC, 2005). Furthermore, since spread of the infection is over much shorter distances in the fish, extensive invasion of the whole host does not require dissemination. Therefore, movement into the bloodstream and spread to other organs likely plays a much less important role in overall virulence in larval fish. Similar results have also been found in a zebrafish model of mycobacterial infection, where comparably extensive mycobacterial growth and pathogenesis occur in both localized hindbrain and bloodstream infections during macrophage ablation (Clay H *et al*., 2007). Due to these differences, we focused on aspects of infection which are most likely to be conserved among small and large vertebrate hosts: basic behaviors of yeast and filaments, and the interplay between morphological forms. These behaviors are likely similar cross-species, in part because of the conservation in the host factors relevant for *C. albicans*-host interaction, such as immune cell types and receptors on epithelial and endothelial cells that mediate endocytosis (Arbizzani F *et al*., 2015, Larson JD *et al*., 2004, Lieschke GJ and Currie PD, 2007, Meeker ND and Trede NS, 2008). Furthermore, the applicability of our study for

mammalian infection is supported by the fact that our findings are consistent with the morphotype-function relationships originally suggested by experiments in mice (Carlisle PL *et al*., 2009, Saville SP *et al*., 2003). These experiments also suggest that filament invasion does not affect the rate of dissemination from tissue-to-bloodstream during mammalian infection. Nevertheless, conservation of basic anatomy and cellular architecture suggests that our findings have potential relevance for mammalian infection.

Experiments with genetic manipulation of five separate *C. albicans* morphology regulators provide strong evidence for a filament-invasion and yeast-dissemination dichotomy. It was important to use several different genetic manipulations, as each individual change could produce non-morphotype-specific artifacts during infection, potentially altering other cellular functions like metabolism and cell wall structural integrity (Murad AMA *et al*., 2001a, Li F and Palecek SP, 2003, Ene IV *et al*., 2014, Polke M *et al*., 2017, Wartenberg A *et al*., 2014). Both wildtype and filament-dominant UME6^{OEX} infections produced rapid invasion and mortality but did not spread through the blood, similar to other zebrafish and mouse infection models (Brothers KM *et al*., 2011, Carlisle PL *et al*., 2009, MacCallum DM and Odds FC, 2005). In contrast, two hypofilamentous mutants (*efg1*Δ/Δ*cph1*Δ/Δ and *eed1*Δ/Δ) and a yeast-locked overexpression strain (NRG1^{OEX}) caused disseminated infection with low mortality, similar to murine infection models (Lo HJ *et al*., 1997, Saville SP *et al*., 2003). Both hypofilamentous mutants grew pseudohyphae at times, consistent with mouse studies observing filamentous growth of the *efg1*Δ/Δ*cph1*Δ/Δ mutant in kidneys (Chen CG *et al*., 2006, Riggle PJ *et al*., 1999). However, similar rates of dissemination and mortality were seen with infections of the morphologically stable NRG1^{OEX} strain, suggesting that pseudohyphae may not play the same role as hyphae. Overall, using these genetic manipulations to alter morphology and monitor the shape/location of all fungal cells in this model provided us with a more thorough understanding of how *C. albicans* utilizes shape to invade tissue and disseminate infection throughout the host.

Temperature manipulations of *C. albicans* morphogenesis in the host provide further evidence for the independent and specialized roles of each morphotype, complementing our results with hypofilamentous strains. These are, to our knowledge, the first experiments using environmental manipulation *in vivo* to perturb and study both yeast and filament contributions to dissemination and invasion in candidiasis. Temperature shifts can alter gene expression and physiology in zebrafish, including responses to oxidative stress and motor activities, and immune responses to infection and wounding can be slowed (Krone PH *et al*., 2003, Lam P *et al*., 2013, Malek RL *et al*., 2004). However, our control experiments monitoring immune homeostasis and wounding responses provide support for the immune stability of zebrafish over short-term infection at temperatures higher and lower than the usual 28°C. While it is possible that other physiological changes at different temperatures may occur, conservation of morphotype-specific activities throughout the range of temperatures suggests that any other physiological changes do not change the specialized activities of yeast and filaments. Taken together with the consistent results in control experiments with shape-locked strains at multiple temperatures, these data suggest that temperature shifts can be used over these time scales to test the functions of *C. albicans* pathogenesis in zebrafish without gross consequences for immune function.

Altering the morphology of the overexpression strains using doxycycline produced similar mortality and dissemination frequencies as the wildtype TT21 infection in zebrafish. In an earlier study, the TT21 strain was found to induce significantly more damage to vaginal epithelium compared to the NRG1^{OEX} strain in a mouse vaginitis model (Peters BM *et al*., 2014). However, this study did not incorporate the use of doxycycline to manipulate morphology of either the TT21 or NRG1^{OEX} strains. Another study using an intravenous murine model implicated a similar strain as our NRG1^{OEX} in infections, and found that overexpression of *NRG1* results in dissemination, but little mortality, while overexpression shut off by doxycycline produced behaviors similar to the filament-dominant wildtype Caf2-1 (Saville SP *et al*., 2003). In our

infections, the addition of doxycycline to TT21 or NRG1^{OEX} also did not significantly alter morphotype production at multiple temperatures, indicating that this drug does not seem to affect the ability of morphology to transition in response to at least one environmental cue. Future studies can incorporate these unique strains by temporally controlling morphology with doxycycline in zebrafish to analyze dissemination and mortality phenotypes at varying times. This control will promote yeast-filament-yeast transitions in real time, allowing us to further understand the potential importance of single-strain dimorphic transitions during invasion and dissemination.

Filament presence moderately correlates with mortality in the zebrafish, but a higher yeast production does not always lead to high dissemination. An increase in filament production led to increased mortality, except for filament-dominant infections at 21°C. This is due to the slowed progression of infection at this temperature, in comparison to 28°C and 33°C. Further, an increase in yeast presence did not necessarily correlate with a direct increase in dissemination. However, our study only incorporates the 30-hour time point from each temperature, in which there are varying degrees of infection progression over time and is not entirely representative of filament function in mortality and yeast function in dissemination. Studies on gastrointestinal dissemination have shown that high levels of yeast must be present for dissemination to occur, and this is increased further after bacterial flora is diminished through antibiotic treatment (Kennedy MJ and Volz PA, 1985, Kennedy MJ and Volz PA, 1983). These studies suggest a hypothesis that a threshold of fungus must be present for dissemination to initiate from the gut (Kennedy MJ and Volz PA, 1985). However, these studies do not decipher between the ratios of yeast and filaments in the gut, and therefore do not answer whether a specific threshold of yeast:filaments is sufficient for dissemination to occur. Future studies analyzing yeast:filament ratios longitudinally during infection in the zebrafish may provide us with a better estimate of when dissemination begins, and a more defined idea of the yeast threshold needed for this initiation.

In human patients, both yeast and filaments of *C. albicans* are found at the infection site, suggesting that the presence of both morphotypes contributes to systemic candidiasis (Nadir E and Kaufshtein M, 2005). Our results show that yeast and filaments have an additive effect on dissemination and mortality during coinfections in zebrafish, providing the first evidence to date for the separability of their functions *in vivo*. Murine infection studies indicate that both yeast- and filament-locked strains have reduced virulence compared to a wildtype that can switch forms (Braun BR *et al*., 2001, Murad AMA *et al*., 2001b, Saville SP *et al*., 2003, Uppuluri P *et al*., 2012, Shen J *et al*., 2008). This suggests that either the presence of both forms or a dimorphic transition is required for virulence. However, there are no studies in mice focused on defined coinfections with yeast- and filament-locked strains, and results from pooled mixed infections suggest that *in vitro* morphology and relative infection progression *in vivo* are not perfectly correlated (Noble SM *et al*., 2010). The additive effect of yeast and filaments on dissemination and mortality during our mixed infections supports the idea that co-occurrence of the two forms is important. Thus, while each form contributes an important component to successful infection, the simple presence of both allows for the important functions of both dissemination and invasion, even if the strains cannot switch back and forth between shapes.

Surprisingly, *C. albicans* morphotypes do not synergize to enhance yeast spread or filament invasion in zebrafish. Interestingly, comparison of mono-infection (filament only) to mixed infection (filaments+yeast) suggests that the presence of yeast during infections results in higher survival (Figures 2.15, 2.16, 2.17), although there was no difference in overall mortality when comparing low versus high yeast inoculum in mixed infections (Figure 2.20). This suggests that the presence of yeast makes a subtle but consistent difference in reducing mortality, but this difference is only associated with the presence or absence of yeast rather than overall yeast inoculum. We do not yet understand how the presence or absence of yeast affects the lethality of the filaments, but this influence could be dependent on other fungal or host factors. For instance, several quorum-sensing factors are known to mediate intercellular

communication among *C. albicans* cells (Chen H *et al*., 2004, Lindsay AK *et al*., 2012). Alternatively, yeast presence could result in immune modulation due to differential immune responses to yeast and filaments (d'Ostiani CF *et al*., 2000, Lowman DW *et al*., 2014). Future studies concurrently analyzing immune response in mixed-morphotype infections may provide us with a better understanding of how yeast affect the ability of filaments to kill fish but not to invade tissue.

Overall, the unique advantages of the zebrafish model described in this study offer a new outlook on host-*C. albicans* interactions in the context of morphological effect on pathogenesis. Interestingly, each form has an additive effect on mortality and dissemination during coinfection, but the presence of both forms does not lead to synergy in pathogenesis. Taken together, these results provide detailed evidence confirming the previously suspected links between shape and pathogenesis, and reveal independent roles for each morphotype during infection. Characterizing the roles of each morphotype during infection has the potential to inform the development and use of promising new morphotype-specific interventions against *C. albicans* (Graham CE *et al*., 2017, Romo JA *et al*., 2017, Vila T *et al*., 2017).

CHAPTER 4

FUTURE DIRECTIONS

Our narrative provides major insight into the roles of yeast and filaments in invasive fungal infections, but leaves out questions regarding fungal cell interactions, host immune interactions, and general mechanisms of action for both dissemination and invasion. Overall, our study has rigorously demonstrated that yeast and filaments confer specialized, but independent, roles during invasive candidiasis in the larval zebrafish, confirming hypotheses from both murine and *in vitro* studies. Further, we find that coinfections with both morphotypes result in an additive effect on dissemination and mortality, but surprisingly do not enhance pathogenesis. These results help shape our ideas of the importance of yeast and filaments during infection, but do not go into detail about what mechanisms might support these roles. Thus, successive work should highlight possible mechanisms through which invasion and damage occur, and how the immune system is affecting these mechanisms and dimorphic transition.

Successive studies should highlight multiple mechanistical questions about dimorphism's role in dissemination, invasion, and host recognition. For instance, during coinfections, we find higher survival when yeast are present, suggesting that factors from either the fungus or the host are affecting this outcome. However, we have not yet dissected which factors might affect the mortality and dissemination phenotypes we observe during these infections. In addition to these results, we are the first study to analyze mixed morphotype infections in the context of mortality and dissemination during invasive infections. However, the long-hypothesized idea that pathogenesis relies on a dimorphic transition between yeast and filaments has yet to be analyzed in this context. We confirm that filament invasion correlates with mortality, and that invasion occurs at the epithelial barriers of the yolk, the heart, and the vasculature. However, we have yet to understand what mechanisms filaments use to cause this invasion and damage to host tissue, although *in vitro* studies have hypothesized multiple mechanisms through which this might occur. Lastly, we find that yeast cells are able to disseminate into the bloodstream, with

and without the presence of filaments. However, we do not yet understand how these yeast cells are crossing epithelial and endothelial barriers to escape into the bloodstream, especially without the assistance of tissue damage by filaments. Overall, this study has provided us with a more thorough understanding of the roles of yeast and filaments during invasive infections in the zebrafish, enhancing our knowledge of each shape's involvement in dissemination. However, this work provides us with even more hypotheses about the underlying mechanisms through which these morphotypes work to cause such invasive infections. Thus, this study lays the groundwork for future studies investigating the detailed mechanisms behind dissemination, invasion, and mortality in invasive candidiasis infections.

In our coinfected fish, we find that yeast presence leads to significantly less mortality, suggesting that yeast may be able to inhibit the ability of filaments to kill. Thus, it is imperative that we analyze which aspects of yeast might be important during this inhibition. In high-density environments, yeast are able to secrete the quorum-sensing molecule farnesol to inhibit filamentous growth from occurring through inhibition of the Ras-cAMP-PKA pathway (Davis-Hanna A *et al*., 2007). It is possible that the yeast-locked strains in our mixed infections could be producing this molecule to slow filament growth. Recently, pravastatin, a drug used to control cholesterol in humans, was found to inhibit farnesol production *in vitro* and improved mouse survival in an intravenous model when used alongside fluconazole (Tashiro M *et al*., 2012). Using this drug to inhibit farnesol production in our zebrafish model could provide insight into whether this molecule is directly related to inhibition of the filament-mortality relationship.

Adhesion between yeast and filaments could potentially alter the ability of filaments to grow or release virulence factors necessary for damage. Thus, testing the ability of cell-cell adhesion mutants could provide us with another explanation for the slowed filament-mortality relationship. The fungal *EAP1* gene is necessary for cell-cell and cell-surface adhesion during biofilm formation *in vitro* and *in vivo*. Produced by both yeast and filaments, a deletion in this gene results in reduced adhesion to other fungal cells, among other surfaces (Li F *et al*., 2007).

Therefore, using a yeast-locked strain lacking *EAP1* in our coinfections could determine whether yeast adhesion to filaments is important for inhibited mortality. However, to our knowledge, there is currently no yeast-locked strain that includes the *EAP1* deletion. Thus, engineering this strain, even with the use of our current yeast strains (NRG1^{OEX}, *efg1*Δ/Δ/*cph1*Δ/Δ) would take time and may not be ideal as a short-term project. Alternatively, using the *eap1Δ/Δ* mutant, which can still form filaments *in vitro*, in conjunction with a yeast-locked strain may provide us insight into whether this adhesin is important for yeast-filament adhesion and pathogenesis *in vivo*. Further, using other adhesion mutants that can germinate, such as the *bcr1Δ/Δ* or *als1Δ/Δ* strains, in conjunction with a yeast-locked strain could also answer whether yeast adherence to filaments is important in the filament-mortality relationship during coinfection (Nobile CJ and Mitchell AP, 2005, Kamai Y *et al*., 2002). Overall, these studies can provide us with insights into the potential impact of secreted fungal chemicals and adhesins on effects of yeast diminishing the ability of filaments to kill fish.

Our study focuses largely on the pathogen aspect of the zebrafish-*C. albicans* interactions, leaving open the potential of host factors in affecting the outcome of the infection. We postulated that the innate immune system could have an impact on the diminished mortality observed in coinfections. In a swimbladder infection model, neutrophils attack and damage filaments with NET formations (Gratacap RL *et al*., 2017). Thus, neutrophils in the yolk could be releasing extracellular traps in the presence of filaments, causing significant damage to filaments and inhibiting their ability to kill the fish. By using an extracellular DNA dye, such as Sytox Green, we could quantify the amount of NET production in each infection and compare between yeast-only, filament-only, and mixed infections to gauge which form provokes the attention of neutrophils in the yolk. Further, cytokine production from an inflammatory response by neutrophils and macrophages to the site of infection could also inform us of the degree of immune response occurring for each morphotype or coinfection, or if there is a difference in cytokine expression in coinfections compared to a presumably inflammatory filament-only

infection. Studies from our lab show that proinflammatory cytokines like IL-6, TNFα, and IL-1β are all induced during *C. albicans* infection in the swimbladder and yolk (Bergeron AC *et al*., 2017, data not published); however, differences in expression during filament-only, yeast-only, and coinfections have yet to be studied. Thus, understanding the differences in immune response between yeast and filaments could give insight into why filaments are less effective at killing, or if filaments are targeted over yeast cells, during these mixed morphotype infections in zebrafish.

Prior studies in mouse infections show that constitutively yeast-locked and filament-locked infections confer less virulence compared to wildtype infections (Saville SP *et al*., 2003, Shen J *et al*., 2008, Murad AMA *et al*., 2001b), suggesting that either the presence of both shapes or a dimorphic switch between shapes during infection is necessary for severe, invasive infection. Our study reveals that the simultaneous presence of both shapes confers an additive effect on both dissemination and mortality, but pathogenesis is left unenhanced. However, we do not completely analyze the dimorphic switch aspect of this hypothesis. By using our tetracycline-regulated overexpression strains, we can temporally alter the shape of a single strain using doxycycline to understand whether dimorphic transition is important for pathogenesis. For instance, we could infect fish with the yeast-dominant NRG1^{OEX} at a filament-inducing temperature, such as 28°C or 33°C, follow fish to a certain time point when dissemination occurs (~24-30 hpi), and then inject doxycycline into the bloodstream or yolk to shut off yeast overexpression and allow filament growth to occur. Similar studies have been analyzed in a mouse intravenous infection with a yeast-locked tet-NRG1 strain and delayed doxycycline treatment to shut off constitutive *NRG1* expression over time, generating a wildtype, filamentous phenotype in organs and mortality (Saville SP *et al*., 2003). Alternatively, we can infect fish with a wildtype strain, promote yeast growth and dissemination at a low temperature, and then increase the temperature to promote filament growth and longitudinally follow mortality over time. These studies, in parallel with yeast-only and filament-only infections, may provide insight

into the importance of single-strain dimorphic transitions for virulent pathogenesis and death in the host.

Many *in vitro* studies have tried to analyze the mechanisms through which filaments invade host tissues and cause damage; however, these have yet to be described *in vivo* due to the opacity of mice involved in the infections. Two major invasion mechanisms, active penetration and induced endocytosis, have been hypothesized to occur during invasion based on *in vitro* studies using epithelial and endothelial cell monolayers (Grubb SEW *et al*., 2008). Thus, our zebrafish model can offer insight into whether filaments use these mechanisms or other mechanisms to confer invasion. Transgenic and protein-fusion fluorescent zebrafish lines for both epithelial and endothelial visualization have long been employed in developmental studies (Trinh LA *et al*., 2011, Lawson ND and Weinstein BM, 2002, Kissa K and Herbomel P, 2010). Using the Tg[*fli1*:EGFP] or Tg[*kdr1*:EGFP] transgenic lines to visualize vasculature and the plakoglobin-citrine protein-fusion line to visualize epithelial tissue, we can visualize filament invasion of these tissues in real time. Similar to mouse studies, we can also use a damage marker, such as dextran microspheres, to follow barrier damage due to filament invasion which may provide us with evidence of the ability of filaments to not only invade tissue, but to cause major damage and leakage of dextran out of the vasculature (Eckle T *et al*., 2008, Zhou G *et al.*, 2012). Further, we can visualize whether filaments actively penetrate host cells or whether host cells initiate invasion via growth of pseudopods for endocytosis using high-resolution time-lapse or time-course microscopy. In addition, quantifying the fluorescence of the fish in images, we can analyze damage of host tissues from filaments (hypothesized less fluorescence), or potential remodeling of tissue during invasion (hypothesized more fluorescence). In conjunction with these fish lines, we can also use adhesin-specific mutant strains of *C. albicans* adhesins that are known to adhere to host tissues *in vitro*, such as *als3*Δ/Δ, *hwp1*Δ/Δ, and *als1*Δ/Δ, to understand which adhesins *C. albicans* uses to initiate invasion, and whether these may also be important for the hypothesized invasion mechanisms described above (Phan QT *et al*., 2007,

Staab JF *et al*., 1999, Sheppard DC *et al*., 2004). These studies can help us identify the exact mechanisms through which filaments cause invasion into epithelial and endothelial barriers in an intact host, and whether the hypothesized *in vitro* mechanisms are adopted *in vivo*.

In our infections, we find that yeast cells are easily able to disseminate from the yolk tissue into the bloodstream and embryonic tissues; however, our work does not investigate the intricate details of how yeast pass through epithelial and endothelial barriers to escape into the bloodstream. We hypothesize that a mix of different mechanisms aid yeast in finding the bloodstream, including phagocytosis and movement in immune cells, endocytosis through endothelial cells, and passage through adherens junctions between host cells. We have visualized groups of yeast cells inside non-fluorescent host cells both inside and outside of the yolk, suggesting that phagocytes, like macrophages and neutrophils, are engulfing yeast and transporting them throughout the body of the fish (data not published). By using transgenic fish with fluorescently-labeled immune cells for macrophages (Tg[*mpeg1*:mCherry]) and neutrophils (Tg[*mpx*:EGFP]) (Ellett F *et al*., 2011, Renshaw SA *et al*., 2006), we can visualize whether or not yeast cells are being engulfed by these immune cells. Prior and current work in our lab has demonstrated that both of these cells are important in phagocytosis of yeast cells and potential dissemination throughout the body (Brothers KM *et al*., 2011, data not published). To discern whether dissemination still occurs in fish without macrophages and neutrophils, a combination of macrophage inhibition using clodronate liposomes in a neutrophil-dysregulated transgenic fish line, Tg[*mpx*:mCherry-2A-*Rac2*^{D57N}], can inhibit both phagocytes (Deng Q *et al*., 2011, Bernut A *et al*., 2014) and dissemination can be longitudinally analyzed. These studies will allow us to test the importance of these two major phagocytes in helping yeast disseminate.

Although prior work shows that phagocytes are important for transport and potential dissemination of yeast cells, yeast transit through host barriers in phagocytes or alone remains unknown. To understand whether endocytosis of yeast cells occurs directly into the bloodstream, we can use the Tg[*fli1*:EGFP] line to visualize vasculature in fish. To directly

analyze endothelial function in uptake of yeast cells, we can ablate both macrophages and neutrophils by using chemical or morpholino inhibition. For instance, dexamethasone, a known corticosteroid, can suppress both macrophages and neutrophils (Voelz K *et al*., 2015). Additionally, injection of the *pu.1* morpholino at the one-cell stage of zebrafish development can inhibit both macrophages and neutrophils for 2-3 days, rendering them immunosuppressed (Knox BP *et al*., 2014). These ablation studies can allow us to visualize whether dissemination still occurs without the two major phagocytes, and whether this dissemination is directly related to endothelial cell endocytosis or passive transport between cells. Current work in our lab has already begun to analyze these aspects, and the results from these studies will provide significant insight into direct mechanisms yeast use to traverse the host tissue barriers and disseminate.

Overall, our study has confirmed and visualized the major roles of yeast and filaments during invasive infections while also providing new insight into how yeast and filaments affect each other during coinfections. However, this study is not an endpoint and raises many more questions that can be investigated by using the zebrafish. Analyzing how yeast and filaments physically interact during coinfections, and how the immune system might affect this interaction are important questions that have not yet been fully addressed. Invasive infections observed in humans show that both yeast and filaments are found in the bloodstream, suggesting that both morphotypes interact to cause these severe infections (Nadir E and Kaufshtein M, 2005). Using the zebrafish to further analyze these interactions, and the interactions and differential responses immune cells may have toward each shape, will help us to better understand these host-pathogen interactions. Further, identifying specific invasion mechanisms filaments use to cause damage, and dissemination mechanisms yeast use to escape into the bloodstream, can also give us new insight into how these deep-seated infections occur, and indicate potential therapeutic targets, on both the host and the fungal sides. Each of these subsequent studies will

provide us with answers to more in-depth host-*C. albicans* interactions that were unable to be visualized prior to the use of the zebrafish infection model.

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APPENDIX: *C. albicans* NEON INFORMATION

CCATGGTTTCAAAAGGTGAAGAAGATAATATGGCTTCATTGCCAGCTACTCATGAATTGCAT
ATTTTTGGTTCAATTAATGGTGTTGATTTTGATATGGTTGGTCAAGGTACTGGTAATCCAAAT
GATGGTTATGAAGAATTGAATTTGAAATCAACTAAAGGTGATTTGCAATTTTCACCTTGGATT
TTGGTTCCACATATTGGTTATGGTTTTCATCAATATTTGCCATATCCAGATGGTATGTCACCA
TTTCAAGCTGCTATGGTTGATGGTTCAGGTTATCAAGTTCATAGAACTATGCAATTTGAAGA
TGGTGCTTCATTGACTGTTAATTATAGATATACTTATGAAGGTTCACATATTAAAGGTGAAG
CTCAAGTTAAAGGTACTGGTTTTCCAGCAGATGGTCCAGTTATGACTAATTCATTGACTGCT
GCTGATTGGTGTAGATCAAAAAAAACTTATCCAAATGATAAAACTATTATTTCAACTTTTAAA
TGGTCATATACTACTGGTAATGGTAAAAGATATAGATCAACTGCTAGAACTACTTATACTTTT
GCTAAACCAATGGCTGCTAATTATTTGAAAAATCAACCAATGTATGTTTTTAGAAAAACTGAA
TTGAAACATTCAAAAACTGAATTGAATTTTAAAGAATGGCAAAAAGCATTTACTGATGTTATG
GGTATGGATGAATTGTATAAATAA**TTAATTAA**

Figure A.1: NEON insert sequence for the pENO1-NEON-NAT^{R} plasmid.
Red, bolded nucleotides represent the flanking restriction enzymes, NcoI and PacI, respectively. Insert size = 715 bp.

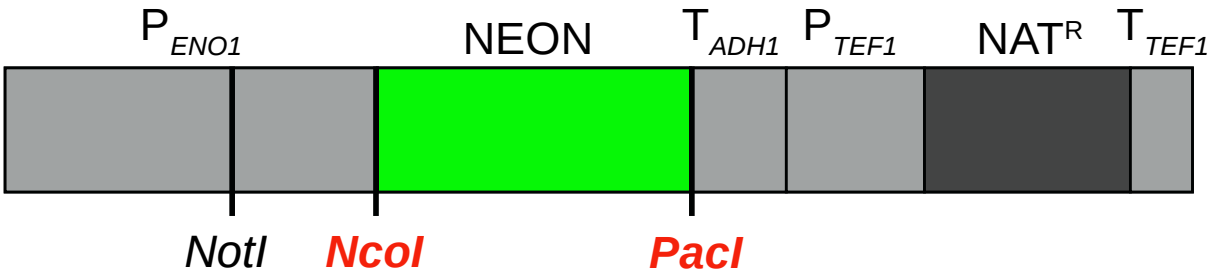

Figure A.2: CUG-optimized NEON *C. albicans*.
The NEON sequence was codon-optimized for CUG-clade organisms and constructed (Shaner NC *et al*., 2013) (GenScript, Piscataway, NJ). NEON expression is under the control of the *C. albicans* constitutive *ENO1* promoter (P_{ENO1}, 1 kb upstream of ORF19.395 from Assembly 21, www.candidagenome.org) and the *ADH1* terminator (T_{ADH1}, GenBank sequence EU493339.1). The transformation plasmid contains the nourseothricin resistance gene (NAT^{R}, dark grey box, GenBank sequence AY854370.1) under the constitutive T_{TEF1} promoter, (P_{TEF1}, GenBank sequence S78175.1) and the TEF1 terminator (T_{TEF1}, GenBank sequence S78175.1) to select viable, transformed colonies. The NotI restriction enzyme was used to stimulate homologous recombination at the *ENO1* locus. Flanking restriction enzymes NcoI (5'-CCATGG-3') and PacI (5'-TTAATTAA-3') are highlighted in red.

BIOGRAPHY OF AUTHOR

Brittany G. Seman was born in Warren, Ohio, on March 6th, 1990. She was raised in Vienna, Ohio where she graduated from Mathews High School in 2008. Following her graduation, Brittany attended Ursuline College for one year on a soccer scholarship, and then transferred to Youngstown State University for her final three years, majoring in Pre-Medicine Biology and graduating in 2012. After earning her Bachelor of Science in Biology, Brittany then continued on to the University of Maine to earn her Doctorate of Philosophy in Microbiology in the Department of Molecular and Biomedical Sciences. She is a candidate for the Doctorate of Philosophy degree in Microbiology from the University of Maine in August 2018.